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Neuron, Vol. 39, 283–297, July 17, 2003, Copyright ©2003 by Cell Press

Selective Regulation of Neurite Extension and Synapse Formation by the β but not the α Isoform of CaMKII

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Summary

Neurite extension and branching are important neuronal plasticity mechanisms that can lead to the addition of synaptic contacts in developing neurons and changes in the number of synapses in mature neurons. Here we show that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) regulates movement, extension, and branching of filopodia and fine dendrites as well as the number of synapses in hippocampal neurons. Only CaMKIIβ, which peaks in expression early in development, but not CaMKIIα, has this morphogenic activity. A small insert in CaMKIIβ, which is absent in CaMKIIα, confers regulated F-actin localization to the enzyme and enables selective upregulation of dendritic motility. These results show that the two main neuronal CaMKII isoforms have markedly different roles in neuronal plasticity, with CaMKIIα regulating synaptic strength and CaMKIIβ controlling the dendritic morphology and number of synapses.

Introduction

One of the remarkable features of many CNS neurons is their ability to construct elaborate axonal or dendritic arbors that can connect them to different input and output neurons. Recent studies reported a significant motility and plasticity of axonal as well as dendritic arbors, suggesting that synaptic contacts change in developing as well as in some mature neurons (Fischer et al., 1998; Jontes et al., 2000; Colicos et al., 2001; Zou and Cline, 1999). Rapid filopodia-like extensions and movements were described in dendritic arbors as necessary steps for neurons to find new contact sites that can then evolve into nascent synapses and mature into functional synaptic connections (Vaughn, 1989; Wong and Wong, 2000; Jontes and Smith, 2000; Ahmari and Smith, 2002). We are investigating in this study the role of CaMKII, a key synaptic signaling protein, in controlling the motility, extension, and branching of dendrites in hippocampal neurons.

CaMKII is a unique neuronal signaling protein that comprises 1%–2% of the protein in brain. It forms oligomers of 12 subunits (Kolodziej et al., 2000). The enzyme is regulated by an intricate activation cascade that involves Ca^{2+}/calmodulin (CaM) binding to each subunit as well as an autophosphorylation step that can render each subunit partially autonomous from Ca^{2+}/CaM binding (Molloy and Kennedy, 1991; Meyer et al., 1992; Hanson et al., 1994; Braun and Schulman, 1995; Hudmon and Schulman, 2002). We found earlier that one can track GFP-conjugated CaMKII in neurons and observe a NMDA receptor-triggered translocation to postsynaptic sites (Shen and Meyer, 1999; Shen et al., 2000), presumably as a result of a binding to the NMDA receptor itself (Bayer et al., 2001). CaMKII has been studied extensively in hippocampal and other neurons as a mediator of plasticity and memory (Silva et al., 1992; Frankland et al., 2001; Malenka and Nicoll, 1999; Malinow and Malenka, 2002; Fink and Meyer, 2002). In a well-supported model for enhancement of synaptic strength, Ca^{2+} entering through NMDA receptor activates CaMKIIα, which in turn leads to an increase in the number (Shi et al., 1999, 2001) as well as the conductivity of AMPA receptors within the postsynaptic membrane (Derkach et al., 1999).

We focused our study on whether CaMKII may not only regulate the strength of synapses but also be involved in regulating the morphology of dendrites. This hypothesis was in part motivated by our previous study where we showed that the second most prominent CaMKII isoform, CaMKIIβ, is localized to the actin cytoskeleton (Shen et al., 1998), positioning it ideally for a role in regulating actin-related morphology processes. Such actin targeting is isoform specific and not found in the predominant neuronal CaMKIIα. While tubulin-based cytoskeletal structures are key components of the main axonal and dendritic branches, most of the cytoskeletal structures in growth cones and extending dendritic processes are driven by actin polymerization (reviewed by Matus, 2000; Meyer and Feldman, 2002). Insights into the actin-based mechanisms that are part of dendritic arbor formation came from pharmacological inhibition studies and fluorescence imaging, as well as genetic studies, which implicated proteins associated with the actin cytoskeleton (Frey et al., 2000; Feng et al., 2000). More recently, the actin-related small GTPases CDC42 and Rac have been linked to the formation of neurite branches, and various external inputs have been shown to enhance or stabilize the dendritic arbor size (Li et al., 2000; Sin et al., 2002; Zipkin et al., 1997; Bito et al., 2000; Fischer et al., 2000; Nakayama et al., 2000; Tanaka et al., 2002; Negishi and Katoh, 2002).

Here we investigate if and how CaMKII isoforms regulate filopodia motility, dendritic branching, and synapse formation in cultured hippocampal neurons. We found that inhibition of CaMKII led to a rapid reduction in motility of filopodia and small dendritic branches and a longer-term decrease in the degree of dendritic arborization. Overexpression of CaMKIIβ, but not the most prominent isoform CaMKIIα, increased the dendritic arborization by a mechanism that can again be suppressed by the same kinase inhibitor. Genetic knockdown of CaMKIIβ levels using RNA interference (RNAi) reduced dendritic arborization and synapse formation,

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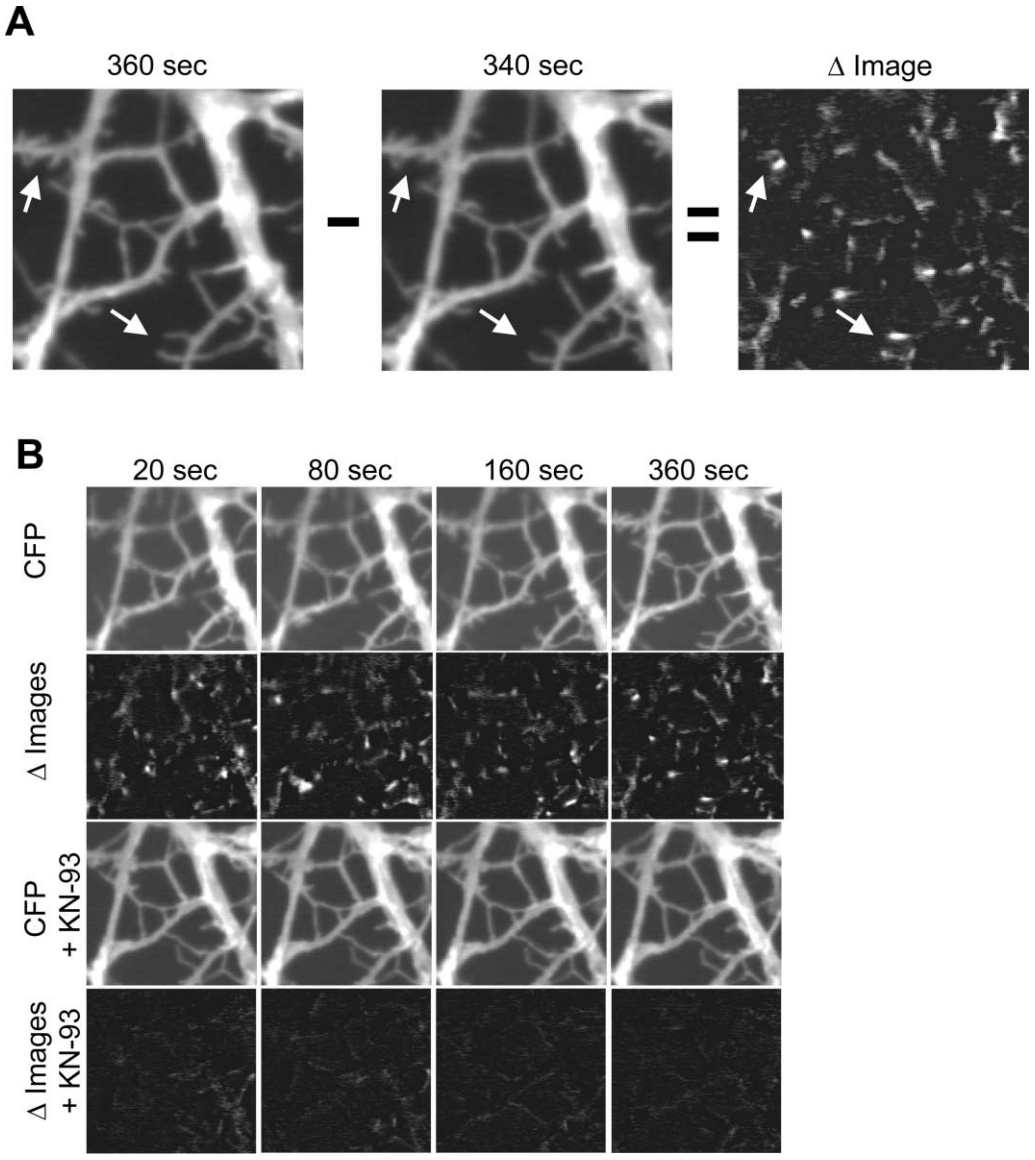

Figure 1. Application of the CaMKII Inhibitor KN-93 Rapidly Slows the Movement of Filopodia and Neuritic Branches

(A) Motility of filopodia and neuritic branches can be observed by difference analysis in a hippocampal neuron, 5 days in vitro (div), and transfected with cytosolic CFP as a fluorescence marker. Images were collected at 20 s intervals. By subtracting an image by the preceding time point in the stack, a difference image is generated, illustrating the movement from one frame to the next. Arrows highlight sample movements of filopodia.

(B) The CaMKII inhibitor KN-93 was applied to the hippocampal neuron shown in (A). Stacks of 20 images were collected at 20 s intervals before and after addition of 10 μM KN-93 (2 min interval between series). The panels show a magnified view of the dotted region in Figure 2A. Rows labeled "CFP" show raw fluorescence data. Rows labeled "ΔImages" show a processed image series in which sequential images were subtracted to show neurite movement.

whereas CaMKIIβ overexpression increased the number of synapses. Analysis of various CaMKII mutants showed that the critical requirement for inducing dendritic movement and arbor extension is a direct binding of CaMKIIβ to polymerized actin. Indeed, in live neurons, actin and CaMKIIβ are both colocalized and enriched at sites of maximal dendritic motility. Together, these data demonstrate a new role for CaMKIIβ in promoting neurite motility and synapse formation.

Results

Rapid Suppression of Neurite Motility and Extension by Inhibition of CaMKII

To investigate the role of CaMKII in neurite extension and branching, we used serial confocal microscopy of primary cultured hippocampal neurons. Neurons, <5 days in vitro (div), were transfected with CFP constructs (Figure 1), and the movement of mostly filopodia-like neu-

rites was analyzed by subtraction of sequential images taken 20 s apart. In this subtraction algorithm, sites where new filopodia extensions appeared or branches moved were positive and sites where fluorescence remained the same or were lost were set to 0 (Figure 1A). Arrows highlight sites where the signal in the difference image results from the movement and formation of filopodia. The many bright areas in series of difference images (Figure 1B, second row) show that there was extensive and ongoing movement of filopodia and small neuritic branches, as well as extension and retraction of filopodia.

We then measured whether the filopodia and branch dynamics changed after addition of 10 μM of the CaMKII inhibitor KN-93. Two minutes after addition, a second time series was collected and difference images were calculated (Figure 1B, third and fourth rows). These images show that the movement and extension of dendrites had largely disappeared in response to the inhibitor. The difference images can be averaged over time and converted to a pseudo-color scale. This allows visualization and quantification of small movements within the different neuronal regions. The motility of filopodia were preferentially decreased at the periphery of the neuritic arbor following addition of KN-93 (Figure 2A; this is a lower magnification of the same neuron shown in Figure 1). Averaged activity changes are shown in Figure 2B in a bar diagram.

The same reduction in motility was observed by measuring the dynamics of individual filopodia. We measured both the net change in filopodia length during a 20 s interval and the sum of all extensions and retractions. A marked difference could be observed in the extension and retraction rates after KN-93 addition as shown in a histogram analysis in Figure 2C. While there was no significant overall change in filopodia length for the two conditions (control, -0.55 ± 1.11 μm; 10 μM KN-93, -1.15 ± 0.71 μm), the sum of the extensions and retractions in control filopodia was much greater than that of filopodia in neurons treated with KN-93 (23.15 ± 1.61 μm versus 11.41 ± 0.82 μm; $p < 0.001$). In addition to these effects on extension and retraction rates, the number of times individual filopodia can been seen to be initiated or to completely retract from main branches significantly decreased in KN-93-treated neurons (Figure 2D) ($p < 0.01$).

As an important control to understand the action of KN-93, we determined whether we could remove the drug to regain filopodia and fine branch motility. As expected from a reversible kinase inhibitor, the washout of KN-93 led to a recovery of most of the motility (Figure 2B). As an additional control, application of the closely related compound KN-92, which does not block CaMKII, did not have an effect on filopodia and fine branch motility.

A concern for even the best kinase inhibitors is their selectivity. KN-93 targets the CaM binding site of CaMKII, but is known to also inhibit CaMKIV, which has been demonstrated to regulate dendritic outgrowth (Redmond et al., 2002). We therefore used an alternative strategy to downregulate CaMKII by using a 27-mer peptide inhibitor, CaM-KIINtide (CNt), that has been derived from a neuronal CaMKII inhibitory protein (Chang et al., 1998) and which we made membrane permeable by the addition of an antennapedia sequence (antCNt). This peptide is highly specific for CaMKII, with little effect on other proteins that KN-93 may also inhibit, such as CaMKI, CaMKIV, CaMKK, protein kinase A, and protein kinase C. Consistent with the hypothesis that CaMKII is necessary for neurite branch activity, the peptide inhibitor led to the same reduction in neurite activity as KN-93, and a control peptide (antCNt-R; reverse sequence) was ineffective (Figure 2B).

Since CaMKII is a Ca^{2+}-activated enzyme, we also measured the effect of removing extracellular Ca^{2+} on neurite activity. This procedure has previously been shown to significantly reduce the fraction of CaMKII that is in the autophosphorylated and autonomously active state (Molloy and Kennedy, 1991). Figure 2B shows that removal of extracellular Ca^{2+} led to a similar loss in motility and extension, consistent with a role of CaMKII in this branch activity. Finally, since CaMKII is a Ca^{2+}/CaM-activated enzyme, we also measured the effect of calmidazolium on branch activity and found that this CaM inhibitor also suppressed neurite motility (Figure 2B).

Expression of CaMKIIβ but not CaMKIIα Increases Dendritic Arborization

Our initial experiments focused on the immediate effect of CaMKII and implicated CaMKII as a critical mediator for the motility of filopodia-like neurite branches. We then determined whether reducing neurite motility by kinase inhibitors has a long-term effect on the fine architecture of the dendritic arbor, particularly filopodia and small dendritic branches. In order to obtain a quantitative measure of dendritic arborization, we measured the perimeter of a neuron expressing a fluorescent protein as marker (Figure 3A). Since we were interested in the degree of fine arborization and not overall dendritic length, a single high-magnification image was taken of the dendritic arbor proximal to the soma. While this analysis does not differentiate between dendrites and axons, the majority of neurites imaged in the field of view are likely to be dendrites. The effect of CaMKII on dendritic arborization was measured for young hippocampal neurons (<5 div) that were treated for 24 hr with KN-93. Untreated control cultures were used as a reference; the average perimeter measurement for these young control neurons was 1032 ± 508 μm (mean ± SD; $n = 90$). The relative changes in branching are shown in Figure 3B for increasing concentrations of KN-93. A dose-dependent decrease in the branch perimeter was observed, with 10 μM KN-93 causing a 60% decrease of the perimeter. Treatment of neurons with KN-92, an inactive analog of KN-93, caused no changes in the perimeter (data not shown).

If CaMKII activity is limiting for dendritic branching, as indicated by the effect of the inhibitor, an increase in CaMKII concentration should increase dendritic branching. Quantitative immunofluorescence allowed us to estimate that we can transfect CaMKIIβ in these neurons at a level that is approximately 4-fold that of the endogenous level (data not shown). We were initially surprised that the expression of the most prominent isoform in the brain, CaMKIIα, had no significant effect on the amount of dendritic arborization (Figure 3C). In contrast,

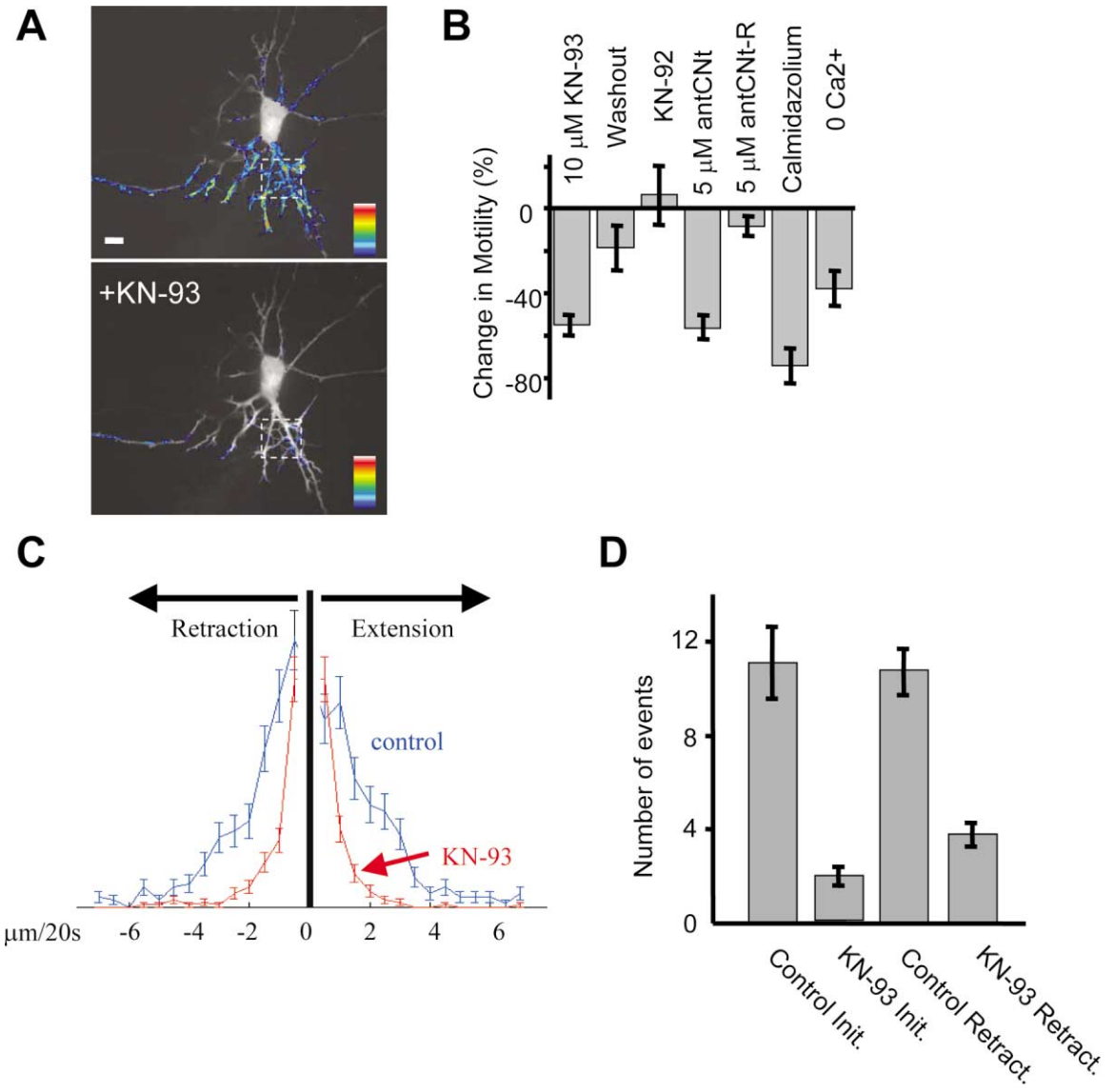

Figure 2. Analysis of Motility Changes in Filopodia and Neuritic Branches of Hippocampal Neurons

(A) Analysis of motility using the neuron shown in Figure 1. The difference images (ΔImages) in Figure 1B were averaged and thresholded and then overlaid over the initial image in pseudo-color. A marked reduction in peripheral filopodida and motility can be seen after addition of the inhibitor. In this particular example, the reduction of motility was 77%. Calibration bar equals 10 μm.

(B) CaMKII inhibitors slow neurite movement. The reduction in total neurite movement is shown after addition of KN-93 (n = 21). Washout of the drug for 15 min allowed for most of the movement to recover (n = 7). Treatment with KN-92 (n = 6), an inactive analog of KN-93, showed no change in movement. Inhibition was also measured after a 15 min treatment with 5 μM of either a specific CaMKII inhibitor peptide (n = 11), or a control peptide with reverse sequence (n = 8). 10 μM calmidazolium (a CaM inhibitor; n = 8) and treatment with extracellular media containing zero Ca^{2+} and 200 μM EGTA for 10 min (n = 6) both caused a significant decrease in motility. The age of all neurons at the time of experimentation is ≤5 div. Change in Motility (%) is plotted as mean ± SEM.

(C) KN-93 decreases filopodia motility. For 9 neurons, the lengths of individual filopodia (46 control and 32 10 μM KN-93-treated) along a selected branch were measured over time. Changes in length for each 20 s time point were calculated and plotted as a histogram (control, blue; KN-93, red). Traces were normalized to each other to account for the difference in n. Error bars represent standard error.

(D) KN-93 decreases the initiation and retraction of filopodia. For the same selected branches analyzed in (C), initiation and retraction events were counted. An initiation event is defined as a new filopodia forming from the dendritic branch and a retraction event is defined as the complete disappearance of a filopodia.

when we expressed the CaMKIIβ isoform, we found a striking increase in dendritic arborization. This indicated that CaMKII-based regulation of dendritic branching is limiting and isoform specific.

The positive effect of CaMKIIβ expression and the negative effect of KN-93 inhibition on the dendritic arbor size enabled us to perform a classical competition experiment. Since KN-93 is a competitive CaMKII inhibitor, its inhibitory effect on dendritic branching should be overcome by expression of more CaMKIIβ and, at much higher drug concentration, such a competition should fail. Figure 3D shows that expression of CaMKIIβ can

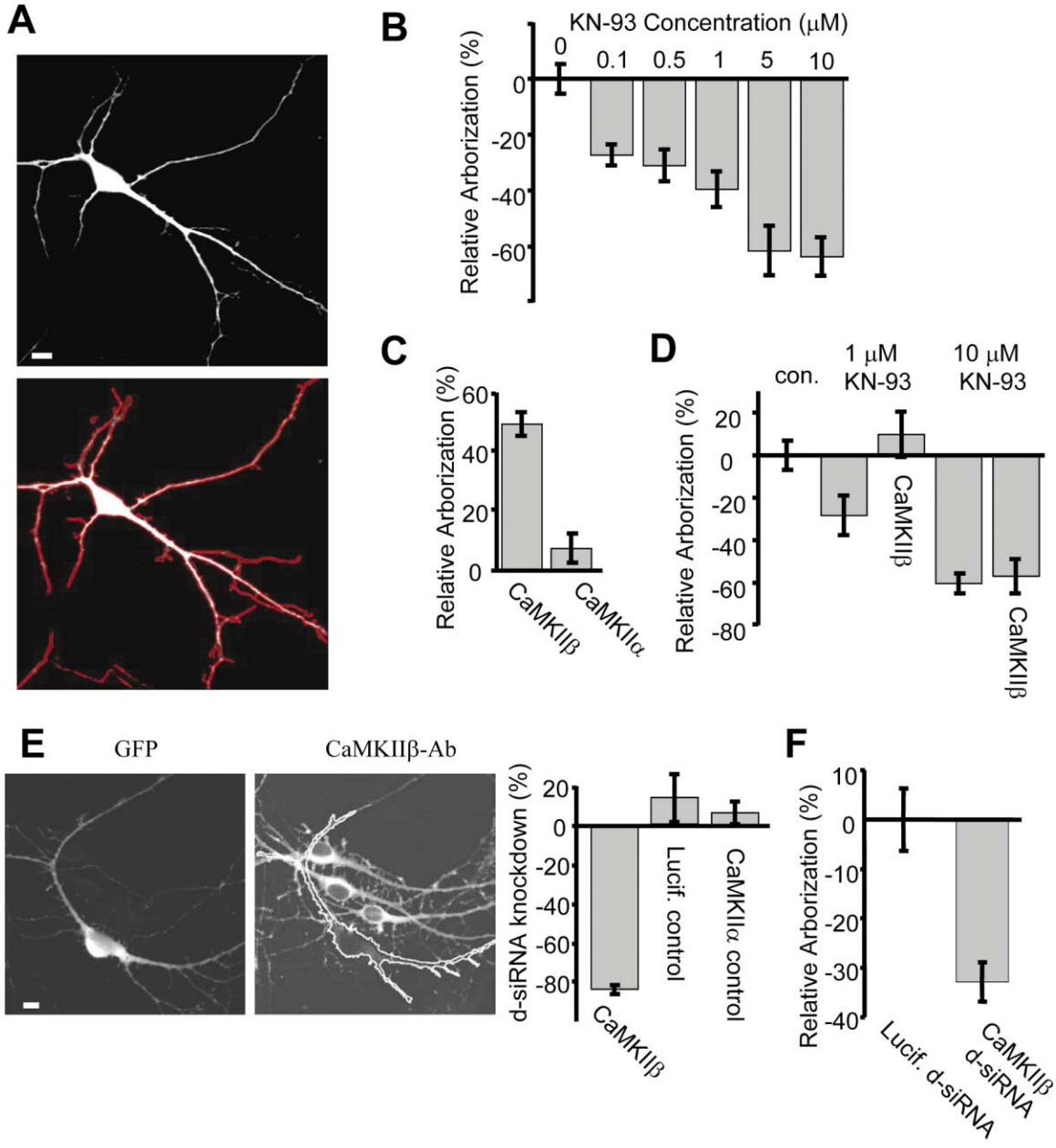

Figure 3. A Selective Role of CaMKIIβ over CaMKIIα in Increasing Dendritic Arborization

(A) Analysis of neurite arbor size by perimeter measurement. Top: hippocampal neurons were transfected with GFP. Bottom: thresholding is used for masking the arbor and the perimeter length was measured using the Metmorph software perimenter function, shown as red. Calibration bar equals 10 μm.

(B) Dose-dependent reduction in the perimenter as a function of KN-93 concentration. Hippocampal neurons (≤4 div) were transfected with GFP and incubated in various concentrations of KN-93 for 24 hr. Concentrations used (in μM) were 0 (n = 27), 0.1 (n = 12), 0.5 (n = 19), 1 (n = 28), 5 (n = 14), and 10 (n = 8). Perimeter values were calibrated against a GFP-transfected control (no KN-93) (mean ± SEM).

(C) Expression of CaMKIIβ, but not CaMKIIα, caused an increase in neuronal branching. Hippocampal neurons (≤4 div) were transfected for 20–28 hr with CaMKIIβ (n = 37) and CaMKIIα (n = 17). For perimeter measurement, images with cotransfected dsRed were used to make sure that the localization pattern of CaMKIIβ did not introduce artifacts into the analysis. This control was performed for all subsequent constructs. Measurements of neuronal perimeters were determined, relative to GFP controls imaged the same day.

(D) Competition experiments between CaMKIIβ expression and CaMKII inhibition. CaMKIIβ transfected into neurons overcomes the effect of 1 μM, but not 10 μM, KN-93. Neurons (≤4 div) were transfected with GFP (n = 12 for the control) or CaMKIIβ (n = 18) and incubated in 1 μM KN-93 for 24 hr. Neurons expressing CaMKIIβ partially reversed the perimeter reduction. This reversal was not observed at 10 μM KN-93 (n = 9 for GFP and n = 9 for CaMKIIβ).

(E) Knockdown of CaMKIIβ with RNAi. 7 div neurons were transfected with GFP (left) and CaMKIIβ (right) d-siRNA for 72 hr. The neurons were fixed and stained with anti-CaMKIIβ (middle). The white outline in the anti-CaMKIIβ panel highlights the location of the GFP-positive (d-si CaMKIIβ) neuron. Calibration bar equals 10 μm. Quantification of CaMKIIβ knockdown was estimated by comparing anti-CaMKIIβ fluorescence intensity of GFP-positive (d-si CaMKIIβ) neurons (n = 16) to controls in the same field of view (right). Neurons transfected for d-si luciferase (n = 12) or stained with anti-CaMKIIα (n = 12) showed no significant change.

(F) RNAi knockdown of CaMKIIβ caused a decrease in dendritic arborization. 5 div neurons were transfected with GFP and either CaMKIIβ d-siRNA (n = 26) or luciferase d-siRNA (n = 25) for 72 hr. Perimeter values were calibrated as the percent change from the luciferase control (mean ± SEM; $p < 0.001$).

indeed reverse the inhibitory effect of a moderate dose of inhibitor without being able to overcome a high dose of the inhibitor.

Inhibition of Dendritric Arborization by d-siRNA-Mediated Reduction in CaMKIIβ Concentration

In order to further strengthen the evidence that CaMKIIβ is involved in regulating arborization, we reduced endogenous CaMKIIβ expression using a novel gene silencing strategy (Meyers et al., 2003). In this method, recombinant dicer protein is used to create a set of double-stranded ~21-mers from a ~500 base double-stranded RNA template that encodes part of mRNA of the protein of interest. When introduced into cells by standard transfection methods, this pool of Dicer-generated small interfering RNA (d-siRNA) provide an effective means to reduce the expression of targeted proteins within ~48–72 hr. Different RNAi strategies have previously been shown to be effective for gene-specific knockdowns in neurons and other cell types (Fire et al., 1998; Elbashir et al., 2001; Hannon, 2002; Krichevsky and Kosik, 2002; Gaudilliere et al., 2002). We demonstrated the efficacy of RNAi in our system by cotransfection of 7-day-old neurons for 72 hr with d-siRNA that target CaMKIIβ together with a plasmid expressing GFP to monitor which cells were transfected. The d-siRNA caused a ~85% knockdown, as assayed by CaMKIIβ immunofluorescence of GFP-positive neurons (Figure 3E). 72 hr was the time point at which peak CaMKIIβ knockdown was observed (data not shown). Transfection with a control firefly luciferase d-siRNA had no effect on CaMKIIβ levels, and CaMKIIβ d-siRNA had no effect on CaMKIIα levels.

We used the genetic knockdown of CaMKIIβ to determine whether the endogenous CaMKIIβ has a limiting role in neuronal arborization. Neurons 5 div were transfected with GFP and CaMKIIβ d-siRNA for 72 hr and imaged to measure neuronal arborization. When compared to control neurons transfected with d-siRNAs targeting luciferase, a 33% decrease in arborization was observed (Figure 3F). These results provide a strong confirmation that CaMKIIβ is critically involved in promoting dendritic arborization.

Developmental Differences in the Role of CaMKIIβ for Neurite Motility and Arborization

Morphogenic activities in dendritic arbors have to accomplish different tasks in developing and mature neurons. In early postnatal development, the predominant mechanisms are the formation of new neurite branch structures and synaptic connections, whereas mature neurons mainly remodel existing dendritic arbors and synapses. We therefore tested the morphogenic function of CaMKII at different stages of neuronal maturation (Figure 4). As described above, overexpression of CaMKIIβ in young hippocampal neurons—cultured for less then 5 days before transfection—significantly enhanced dendritic arborization. Figure 4A shows that this positive effect of CaMKIIβ expression became smaller and even negative as the neurons mature.

Since the long-term limitation of arborization in mature neurons is likely dependent on multiple activity- and contact-dependent negative control mechanisms, we performed experiments using inhibition of the endogenous enzyme and live imaging of filopodia motility and extension to measure whether CaMKII inhibition has immediate effects on filopodia. While the overall filopodia-like motility becomes smaller as neurons establish a more permanent arbor, the remaining movement and extension of filopodia-like branches was still markedly reduced by CaMKII inhibition (>13 div). This loss of filopodia activity in mature neurons can best be seen in the difference images in Figure 4B and in a bar diagram in Figure 4C. These results indicate that CaMKIIβ plays a critical morphogenic role in mature neurons, but now in permitting the remodeling of existing arbors rather than in increasing the degree of arborization.

A possible explanation for the loss of an effect in CaMKIIβ expression on arborization in mature neurons is that the native CaMKIIβ concentration may not be limiting any more due to increased expression of the endogenous enzyme. Indeed, while Western blot analysis shows the presence of CaMKIIβ as early as 1 day after plating, CaMKIIβ expression markedly increased around day 5 and reached a plateau only 7–8 days after plating. It is also interesting to note that CaMKIIβ remained the predominant isoform until day 9 (Figure 4D). While small amounts of α-isoform can be detected in the hippocampal neurons already in the first day after plating, expression of the CaMKIIα becomes predominant only around day 10 and plateau levels are reached at day 17. Overall, these relative expression patterns in cultured neurons are consistent with previous studies on relative CaMKIIα and CaMKIIβ RNA levels in the developing brain (Bayer et al., 1999).

Increases and Decreases in CaMKIIβ Concentration Lead to Corresponding Increases and Decreases in the Number of Synapses

What is the physiological relevance of increased filopodia motility and fine dendritic branching? It has been hypothesized that filopodia motility is required for the establishment of synapses (Vaughn, 1989; Wong and Wong, 2000; Jontes and Smith, 2000; Ahmari and Smith, 2002). We therefore tested the hypothesis that CaMKIIβ levels may directly control the number of synapses. PSD95-YFP was used as a postsynaptic marker to count the number of synapses in a fixed dendritic area around the cell body. 14 div neurons transfected with PSD95-YFP show large numbers of punctate spots in the dendritic arbor (Figure 4E, left). In the first experiment, neurons were transfected at 7 div with PSD95-YFP and CaMKIIβ-CFP to increase the total concentration of CaMKIIβ. Cotransfection of PSD95-YFP with Lyn-CFP was used as a control. Cells were imaged at 14 div, and PSDs were manually counted. Consistent with the hypothesis that CaMKIIβ concentration controls the number of synapses, neurons transfected with CaMKIIβ had 40% more PSDs/μm^2 than the control neurons (Figure 4E, middle; $p < 0.01$).

To further test this intriguing hypothesis, we performed the inverse experiment by silencing the expression of CaMKIIβ. Neurons 7 div were cotransfected with CaMKIIβ d-siRNA and PSD95-YFP and the number of

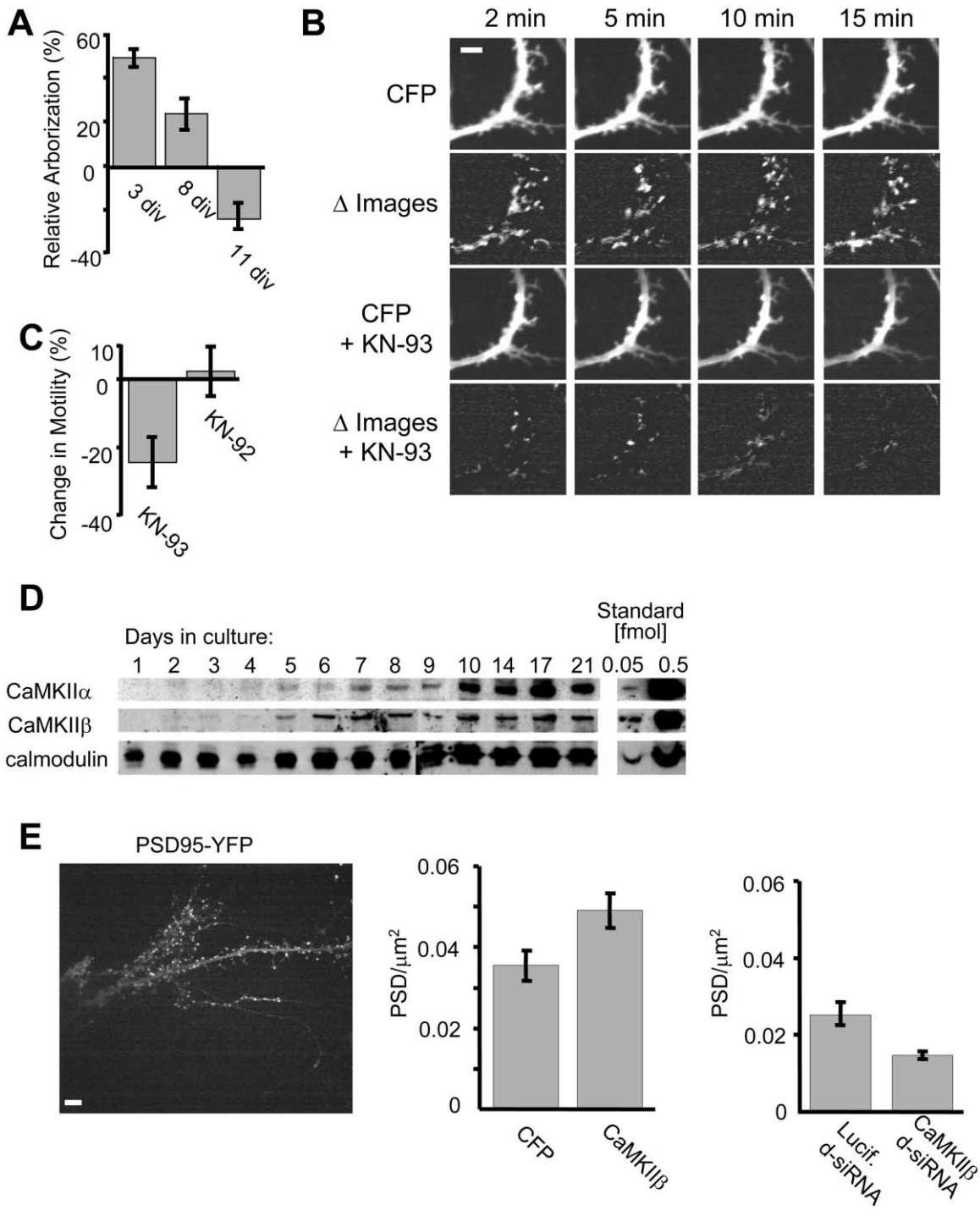

Figure 4. CaMKIIβ Plays a Role in Mature Neurons Promoting Filopodia Motility and Synapse Formation

(A) The ability of CaMKIIβ to promote dendritic arborization decreases with age. Neurons transfected with GFP-CaMKIIβ were transfected for 20–28 hr and imaged 3 div (n = 17), 8 div (n = 16), and 11 div (n = 9). Perimeter change from GFP-transfected control is plotted (mean ± SEM).

(B) 10 μM KN-93 slowed the movement of filopodia and small dendritic branches in a hippocampal neuron (13 div) transfected with CFP-GPI as a fluorescent marker. As in Figure 1B, series of original and difference images (taken every 60 s) are shown before and after KN-93 addition. Calibration bar equals 2 μm.

(C) KN-93 slows movement of filopodia and dendritic branches in mature (≥13 div) hippocampal neurons (n = 10), while addition of KN-92 has no effect (n = 21). Change in Motility (%) is plotted as mean ± SEM.

(D) Western blot data shows the time-dependent expression of CaMKIIα and CaMKIIβ (top lines) in cultured hippocampal neurons. CaM expression (bottom line) is shown as a control. Purified proteins were used as standards.

(E) CaMKIIβ promotes synapse formation. Left: a 14 div neuron transfected with PSD95-YFP. Calibration bar equals 10 μm. Middle: 7 div neurons were transfected with PSD95-YFP and either CFP (n = 24) or CaMKIIβ-CFP (n = 23) and imaged at 14 div. Individual PSDs were counted and plotted per unit area (mean ± SEM; $p < 0.01$). Right: 7 div neurons were transfected with PSD95-YFP and either CaMKIIβ siRNA (n = 19) or luciferase siRNA (n = 16) for 72 hr. Individual PSDs were counted and plotted per unit area (mean ± SEM; $p < 0.01$).

PSD95 marked synapses were counted at 10 div. Cotransfection of luciferase d-siRNA and PSD95-YFP was used as a control. Strikingly, neurons transfected with CaMKIIβ d-siRNA had 40% fewer PSDs/μm^2 than the luciferase controls (Figure 4E, right; $p < 0.001$), suggesting that the concentration of CaMKIIβ is directly involved in controlling the number of synapses.

CaMKIIβ but not CaMKIIα Binds to Polymerized Actin

What is the mechanism underlying the isoform specificity of the morphogenic CaMKII activity in neurons, especially in light of the similar broad substrate specificity of CaMKIIα and β? As described previously (Shen and Meyer, 1999), GFP-labeled CaMKIIα is largely cytosolic when expressed in hippocampal neurons, whereas CaMKIIβ shows a cortical actin localization (Figure 5A). These earlier studies also indicated that a direct interaction of CaMKIIβ with F-actin is involved in this subcellular distribution. In order to examine the possible interaction of CaMKII with F-actin in more detail, we performed biochemical binding assays using purified proteins (Figure 5B). Indeed, purified CaMKIIβ but not CaMKIIα bound to polymerized actin. This interaction was disrupted by Ca^{2+}/CaM, but not by either Ca^{2+} or CaM alone. This can explain how GFP-CaMKIIβ dissociates from actin stress fibers in transfected cells upon a Ca^{2+} stimulus (Shen and Meyer, 1999).

This raises the question whether CaMKIIβ could be directly activated by actin binding without a requirement of Ca^{2+}/CaM, as was shown previously for the interaction of CaMKIIα with the NMDA receptor subunit NR2B (Bayer et al., 2001). We tested this possibility in a biochemical phosphorylation assay using purified protein and a peptide substrate (Figure 5C). Unlike NR2B binding, binding of CaMKIIβ to F-actin did not lead to increased activity toward the soluble peptide substrate; only basal activity of less then 1% compared to the maximal Ca^{2+}/CaM-stimulated activity was observed. This suggests that Ca^{2+}/CaM binding to some of the oligomeric subunits is needed to obtain actin-localized kinase activity.

We investigated the predicted colocalization of actin and CaMKIIβ in live neurons by cotransfecting CFP-conjugated CaMKIIβ and YFP-conjugated actin. As shown in Figure 6A, there is near complete overlap within the sites in the dendritic arbor that have enriched actin and the sites that have enriched CaMKIIβ. An analysis of neurite motility and extension showed a marked correlation between the sites where CaMKIIβ and actin are enriched and the sites where neurite movement and extension occurs (Figures 6B and 6C). Thus, CaMKIIβ is in the best possible position to locally regulate neurite extension and branching.

In experiments in which actin was rapidly depolymerized using cytochalasin-D (10 μM), a marked suppression of actin-labeled dendritic motility was observed that had the same kinetics as addition of CaMKII inhibitors (Figure 6D). This similar kinetics is consistent with a direct role of CaMKII in regulating actin polymerization. Interestingly, only a strong Ca^{2+} stimulus, either by glutamate or ionomycin, but not the partial depolymerization induced by cytocholasin-D, did lead to a loss in colocalization of CaMKIIβ and actin (data not shown). This is consistent with the biochemically characterized competition between binding of CaMKIIβ to Ca^{2+}/CaM and actin (see Figure 5B).

Actin Binding Is Required for the Morphological Activity of CaMKIIβ

In order to better understand whether binding of CaMKIIβ to actin is required for its role in neurite motility and neurite branching, we tested the effect of several CaMKII mutants. A chimeric GFP-CaMKII construct was made, consisting of GFP-CaMKIIα catalytic, regulatory, and oligomerization domains but with the short variable region of CaMKIIβ inserted between the regulatory and the oligomerization domain (Figure 7A). When this CaMKIIα/β chimera was expressed in neurons, it had a partial actin localization (Figure 7B) that was less pronounced when compared to CaMKIIβ (see Figure 5A, right). Consistent with the hypothesis that actin localization is key to functional specificity, the chimeric CaMKIIα now induced a significant increase in the size of the dendritic arbor (Figure 7C). The increase mediated by the chimera was less pronounced than for CaMKIIβ and is thus well correlated with the degree of actin localization.

When we tested a constitutively active mutant of CaMKIIβ (T287D; mimicking autophosphorylation) for its effect on dendritic arborization, we also observed a significant increase (Figure 7D), but to a lesser extent when compared to the wild-type. Again, the smaller effect of the T287D mutant is correlated with its less pronounced actin localization (Figure 7E, left), further indicating that localization of the kinase is an even more important limiting factor than kinase activity. We next examined two CaMKIIβ mutants with impaired activation (A303R; impaired CaM binding) or activity (K43R; impaired ATP binding). Again, the effect on neurite outgrowth is highly correlated to the actin localization. Interestingly, the A303R mutant had a similar positive effect as wild-type despite its impaired activation (Figure 7E, right). This mutant has an actin localization that is persistent, as it cannot be reversed by CaM binding. The mechanism for enhancing dendritic arborization may involve permanent cotargeting of endogenous CaMKII (by forming hetero-oligomers) that can still be activated by Ca^{2+}/CaM. By contrast, the K43R mutant has a negative effect on degree of arborization. This mutant has a similar localization as wild-type (data not shown), and thus would likely compete with endogenous CaMKII rather than anchoring it, thereby causing a dominant-negative effect.

Finally, we tested the doubly inactivating mutant A303R/K43R, which has an enhanced actin binding but a reduced Ca^{2+}/CaM binding as well as an inactivated kinase catalytic site. The effect of this double mutant was intermediate, showing a significant increase in dendritic arborization, but not as pronounced as observed for wild-type or the A303R mutant. Thus, the negative effect of the inactive K43R mutant can be "rescued" by a mutation that leads to stronger actin association. Furthermore, this also implies that the pronounced positive effect of the A303R mutant is in part due to increased targeting of endogenous wild-type CaMKII.

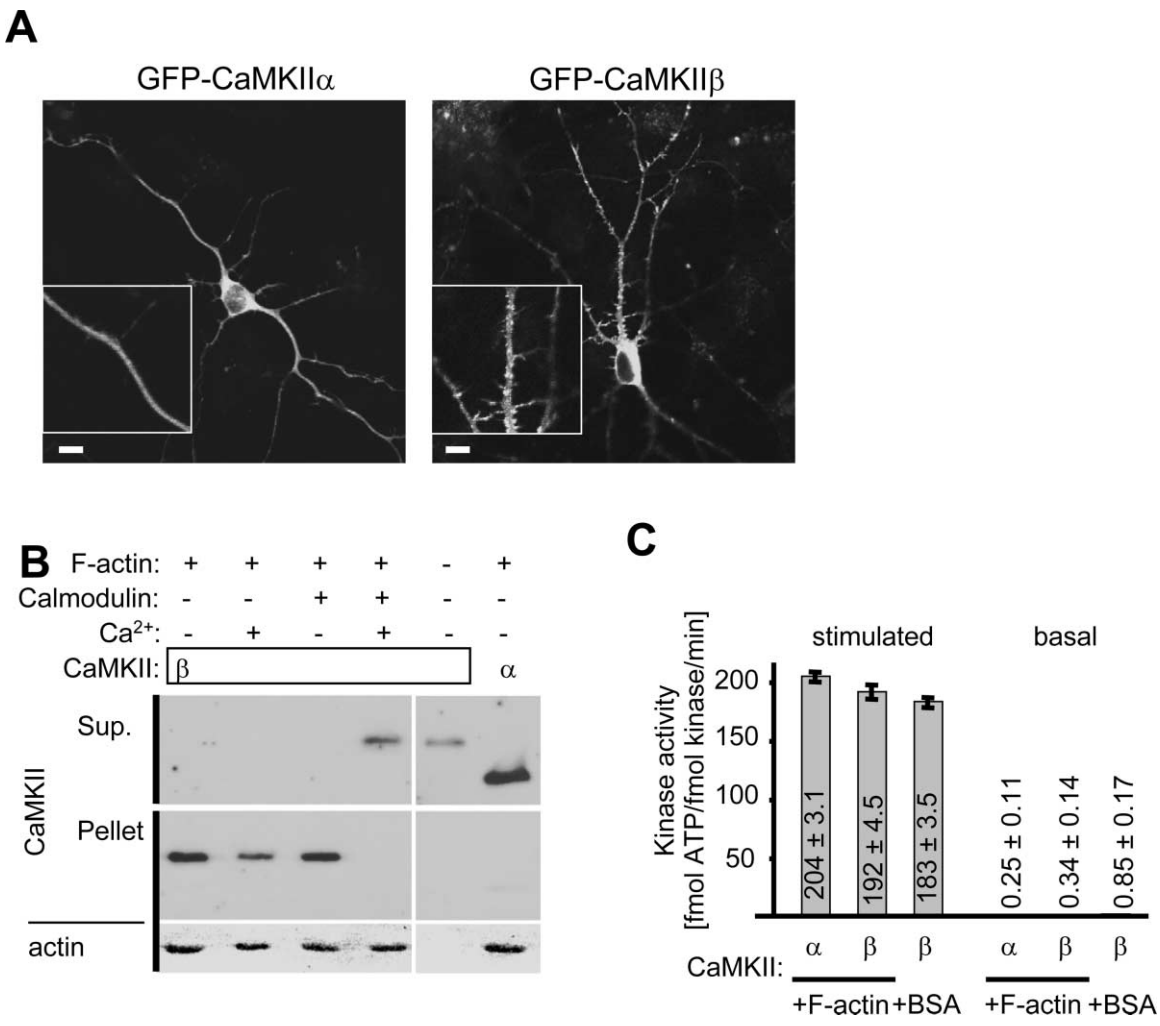

Figure 5. CaMKIIβ, but not CaMKIIα, Binds to Polymerized Actin In Vitro and in Live Cells

(A) Confocal image of GFP-tagged CaMKIIα (left) and CaMKIIβ (right) in living hippocampal neurons (≤7 days after plating). The inset in the lower left corner of each panel is a magnified view. While GFP-CaMKIIα has a uniform cytosolic distribution, GFP-CaMKIIβ has an actin-like localization. Calibration bars equal 10 μm.

(B) CaMKIIβ, but not CaMKIIα, binds to F-actin in the absence of Ca^{2+}/CaM. CaMKIIβ copellets with F-actin in a biochemical assay with purified protein. The interaction is disrupted in the presence of Ca^{2+} and CaM, but not by either one alone. The CaMKIIα isoform is found in the supernatant. CaMKII and actin were detected by calmodulin overlay or Ponceau S staining of the blotted fractions, respectively.

(C) Binding of CaMKIIβ to actin does not significantly increase the kinase activity. CaMKII activity can be stimulated by Ca^{2+}/calmodulin (1 mM/1 μM) in presence of F-actin (left), as determined by a phosphorylation assay using the peptide substrate AC3. In nonstimulated basal conditions (without Ca^{2+}/calmodulin), F-actin does not induce autonomous activity of CaMKII (right). Data points are means ± SEM.

Discussion

Neurite extension and dendrite remodeling are important mechanisms for neuronal development and plasticity. Our study shows that the β but not the α isoform of CaMKII enhances filopodia extensions and dendritic motility. In developing hippocampal neurons, this CaMKIIβ activity promotes arborization of the dendritic tree. In mature neurons, CaMKIIβ still has a strong morphogenic effect, but now promotes dendritic remodeling rather than overall arborization. Consistent with the hypothesis that filpodia extension and motility are key processes required for synapse formation, our study showed that CaMKIIβ significantly increased the number of synapses when expressed ~4-fold above wild-type and that CaMKIIβ knockdown by d-siRNA markedly reduced the number of synapses. Complementary experiments using CaMKII inhibitors, overexpression of various CaMKII constructs, and genetic knockdown using RNAi showed that CaMKIIβ activity is a limiting factor for dendritic motility and branching. In addition, we found that targeting of CaMKIIβ to F-actin is critical for this activity, explaining that only the actin-localized CaMKIIβ but not CaMKIIα is capable of mediating this morphological activity.

A strong argument that CaMKIIβ has a direct role in regulating dendritic morphology is the near immediate effect that CaMKII inhibition has on filopodia extension and motility in developing and adult neurons. Such a direct link is further supported by the observed marked

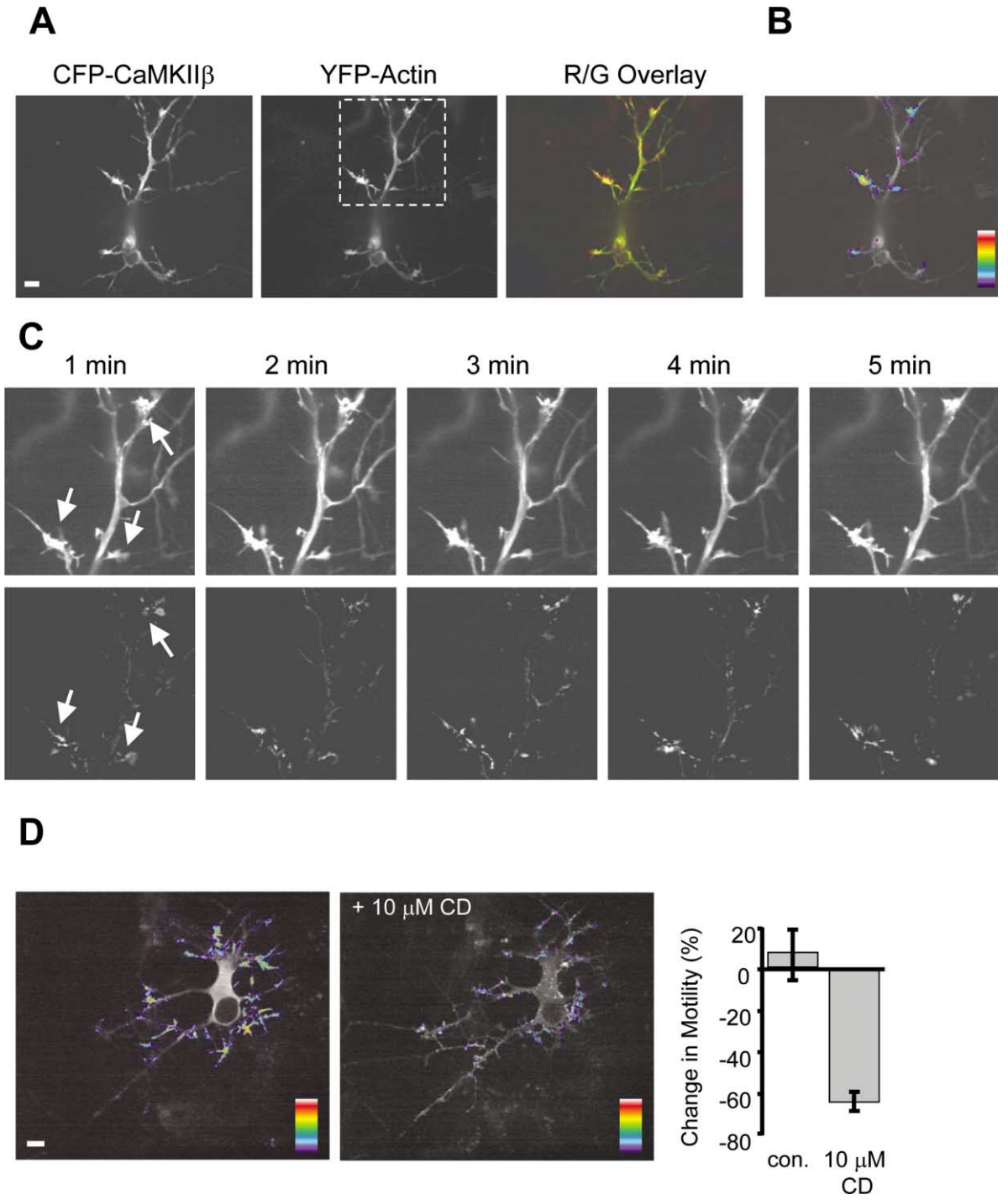

Figure 6. CaMKIIβ Localizes to Sites of Filopodia Initiation and Neurite Motility

(A) CaMKIIβ and actin colocalize in young neurons. A hippocampal neuron (4 div) was cotransfected with CFP-CaMKIIβ and YFP-actin for 16 hr, and confocal images were collected. Both CFP-CaMKIIβ (left) and YFP-actin (middle) show a strong punctate staining pattern. Control experiments using coexpressed cytosol-localized constructs showed no punctate staining (data not shown). An overlay of both images (right) shows near complete colocalization. Calibration bar equals 10 μm.

(B) The neuron from (A), with an overlaid pseudocolored motility image, is shown for the time series.

(C) Active filopodia movement is initiated from sites enriched in CaMKIIβ and actin. For the neuron shown in (A), a magnified view of the YFP-actin fluorescence (dotted rectangle, middle panel of A) is shown over time (20 images were collected in the time series, at 20 s intervals). The top row represents raw fluorescence data, while the bottom row illustrates the difference data. The arrows in the first panel (1 min time point) highlight three regions of high motility that correlate with actin/CaMKIIβ localization.

(D) Depolymerization by 10 μM cytochalasin-D slows actin-labeled dendritic motility. A sample neuron with an overlay of pseudocolored movement is shown for a time series before (left) and after (right) treatment with cytochalasin-D. Actin and CaMKIIβ remain colocalized as polymerized actin is fragmented. The Motility Index for all neurons (≤5 div) treated with cytochalasin-D ($n = 10$) or a control solution change ($n = 6$) is plotted on the right as mean ± SEM. Calibration bar equals 10 μm.

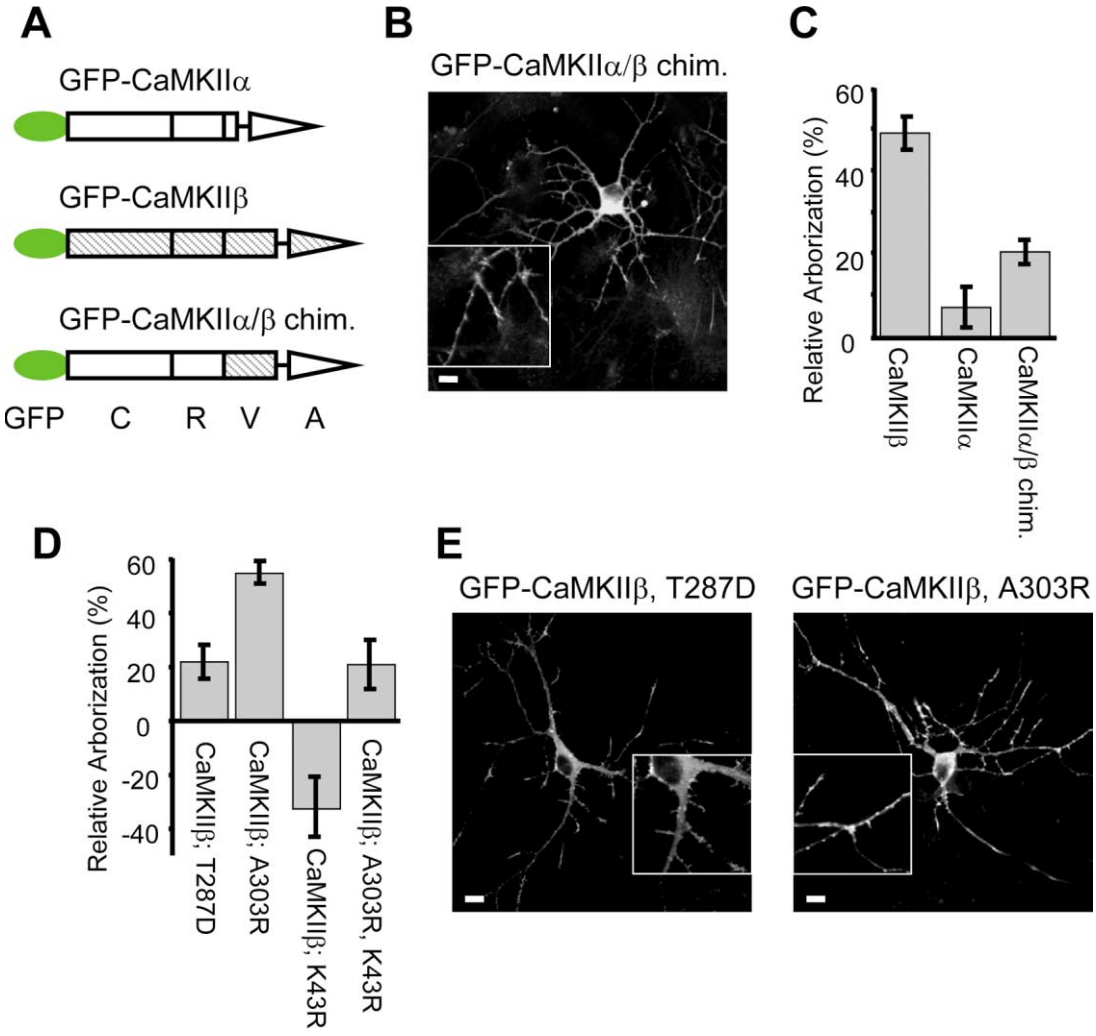

Figure 7. Binding of CaMKIIβ to Actin Is Critical for Its Selective Role in Regulating Arbor Formation

(A) Schematic representation of the domain organization of the GFP-tagged CaMKII isoforms. The catalytic domain (C), regulatory domain (R), variable domain (V), and oligomerization domain (A) are shown. A chimeric CaMKII was constructed by exchanging the variable domain of CaMKIIα with the β-specific variable domain.

(B) Confocal image of GFP-tagged CaMKIIα/β chimera in a living hippocampal neuron (≤7 div). The inset in the lower left corner is a magnified view. GFP-CaMKIIα/β chimera has a weaker actin colocalization than GFP-CaMKIIβ. Calibration bar equals 10 μm.

(C) Measurements of neurite perimeters for wild-type and chimeric GFP-CaMKII. Hippocampal neurons (≤4 div) were transfected for 20–28 hr. Constructs examined were CaMKIIβ (n = 37), CaMKIIα (n = 17), and CaMKII α/β chimera (n = 34). For these experiments, only neurons of approximately the same fluorescent intensity were chosen for analysis. For perimeter measurement, images with cotransfected dsRed were used to make sure that the localization pattern of CaMKIIβ did not introduce artifacts into the analysis. Data expressed as mean ± SEM.

(D) Changes in arbor morphology induced by expression of mutant CaMKIIα and β. Hippocampal neurons (≤4 div) were transfected for 20–28 hr. Constructs examined were CaMKIIβ T287D mutant (n = 19), CaMKIIβ A303R mutant (n = 43), CaMKIIβ K42R mutant (n = 16), and CaMKIIβ K42R/A303R mutant (n = 16). Data expressed as mean ± SEM.

(E) Confocal image of GFP-tagged CaMKII β T287D mutant (left) and CaMKIIβ A303R mutant (right) in living hippocampal neurons (≤7 div). The inset is a magnified view. GFP-CaMKIIβ A303R has a strong actin-like localization while GFP-CaMKIIβ T87D has a weaker actin colocalization than GFP-CaMKIIβ. Calibration bars equal 10 μm.

enrichment of CaMKIIβ at the sites where filopodia extension occurs. We show that this enrichment is the result of CaMKIIβ binding to polymerized actin that is present at these same initiation sites, indicating that CaMKIIβ takes part in the positive feedback process for filopodia extension. While the list of the potential effectors of CaMKIIβ includes regulators of the small GTPase family and other targets, the large number of known actin-related proteins that interact with CaMKII such as α-actinin (Walikonis et al., 2001) and caldesmon (Ikebe and Reardon, 1990) suggests that CaMKIIβ's role in cytoskeletal restructuring may involve phosphorylation of multiple targets.

Molecular Mechanisms of CaMKIIβ Activation

CaMKIIα and β have a similar and broad substrate selectivity when tested in in vitro assays (Braun and Schulman, 1995). We found in this study that localization of CaMKII activity to the actin cytoskeleton is the main reason why CaMKIIβ and not CaMKIIα is regulating

dendritic morphology. Indeed, when we inserted a short CaMKIIβ-specific part of the variable region into CaMKIIα, it conferred a partial actin colocalization to this chimeric enzyme that was paralleled by a partial effect on the dendritic morphology. The importance of targeting is further highlighted by the fact that the morphogenic effect of various CaMKIIβ mutants was more strongly correlated with the degree of actin localization than with activity. The specificity between CaMKII isoforms is therefore a result of differential subcellular localization and not of differential substrate specificity.

How is CaMKIIβ activated? An earlier study showed that the actin binding interaction of CaMKIIβ is reversible and involves a competitive binding between actin and Ca^{2+}/CaM (Shen and Meyer, 1999). We therefore tested the possibility that actin binding itself may enhance activity of CaMKIIβ, as has been demonstrated previously for CaMKIIα bound to the NMDA receptor subunit NR2B (Bayer et al., 2001). In our biochemical analysis, binding of CaMKIIβ to F-actin did not induce any activity toward a soluble peptide substrate. Instead, activation of CaMKIIβ required Ca^{2+}/CaM and dendritic motility required Ca^{2+} and CaM signals as well as Ca^{2+}/CaM binding to CaMKII. This was demonstrated by using the corresponding inhibitors EGTA, calmidazolium, and KN-93, respectively. However, it should be noted that Ca^{2+}/CaM present in unstimulated neurons was sufficient for mediating CaMKIIβ-dependent dendritic extension and motility. Thus, the Ca^{2+}/CaM stimulus needed was readily provided by the basal neuronal activity in the cultures, suggesting that the morphogenic function of CaMKIIβ only requires relatively weak Ca^{2+}/CaM signals. Such requirement for only basal neuronal activity is consistent with the earlier finding that 25% of CaMKII is autophosphorylated at T286/287 already under basal conditions (Molloy and Kennedy, 1991). Since autophosphorylation requires Ca^{2+}/CaM binding, this implies that sufficient Ca^{2+}/CaM is present at basal neuronal activity for partial CaMKIIβ activation.

Biochemical studies showed that CaMKIIβ is activated by Ca^{2+}/CaM at a 4- to 5-fold lower concentration than the α-isoform (Brocke et al., 1999), suggesting that CaMKIIβ is favored over CaMKIIα for low Ca^{2+}/CaM signals. Since only about two β subunits are needed for actin binding, low to intermediate levels of Ca^{2+}/CaM should activate the enzyme while it is still bound to actin, suggesting that actin-localized proteins are preferential targets for CaMKIIβ as long as Ca^{2+} levels are not too high. High concentrations of Ca^{2+} can lead to a competition between Ca^{2+}/CaM and CaMKIIβ for actin binding and result in a complete dissociation of CaMKIIβ from the actin cytoskeleton (Shen and Meyer, 1999). Therefore, the regulatory effect of CaMKIIβ on cytoskeleton architecture is predicted to be optimal in basal to intermediate ranges of Ca^{2+} concentration.

Roles of CaMKIIα versus CaMKIIβ

Together, our findings suggest a model by which low-level stimulation preferentially activates CaMKIIβ, leading to increased filopodia extension and synapse formation. In contrast, CaMKIIα would require additional stronger stimulation in order to enhance AMPA-type glutamate receptor currents. This would suggest that not only the roles of CaMKIIα and CaMKIIβ but also their activation signals are markedly different.

These differential roles of the two CaMKII isoforms suggest that different levels of expression of these isoforms lead either to a strengthening of the synapse if CaMKIIα function dominates or filopodia extension and synapse formation if CaMKIIβ dominates. Consistent with a homeostatic role of this dually regulated mechanism, the α/β ratio was found to change by ~5-fold depending on the stimulation state of a hippocampal neuron (Thiagarajan et al., 2002). The α/β ratio shifts toward α when the neurons are stimulated and toward β when the neurons are pharmacologically depressed. Furthermore, Wu and Cline (1998) have shown that overexpression of CaMKIIα can lead to dendritic stabilization. Together with our findings, one can postulate that high-stimulation conditions increase the relative CaMKIIα expression and that the relative reduction in CaMKIIβ would limit the number of synapses. In contrast, the low-stimulation conditions would increase the relative CaMKIIβ expression, which in turn would upregulate synapse formation and thereby increase the total inputs into the neurons. This dual control mechanism can provide a powerful homeostatic control switch to keep the overall neuronal inputs in a desired activity window.

These considerations predict that neurons with a persistently high presence of β isoforms would have a high degree of arborization and large numbers of synapses. As an example, cerebellar neurons have persistently high CaMKIIβ level with a relative β to α ratio that is ~12 times higher than in neurons in the forebrain (Miller and Kennedy, 1985). Consistent with a morphogenic role of CaMKIIβ, cerebellar neurons have a highly branched morphology compared to those in the forebrain, where the β to α ratio is small.

In further support for such a morphogenic role of CaMKIIβ, CaMKIIβ is also the isoform expressed earlier in development compared to CaMKIIα (Bayer et al., 1999), during the time when neurons have to build up their dendritic arbor. Even in hippocampus, the brain region with the earliest onset of CaMKIIα expression and the highest expression level in the mature brain, CaMKIIβ dominates at least during the first postnatal week.

One can also predict a second plausible mechanism by which the relative ratio of expressed CaMKIIα and β can control neuronal plasticity. The coexpression of the α and β isoform has been shown to lead to stochastically mixed oligomers that can still colocalize with actin as long as two or more β subunits are present in an α/β hetero-oligomer (Shen et al., 1998). Since the mRNA for CaMKIIα but not for β can be found in the dendritic arbor, it is likely that the ratio of local translation in dendrites versus cell body defines the relative number of actin-localized CaMKII activity. The protein translated in dendrites would primarily create CaMKIIα-homomers that are not actin localized and regulate synaptic strength while the mixed fraction translated in the cell body would create α/β hetero-oligomers that primarily that bind actin and regulate filopodia extension and synapse formation. Such a translational control of actin-localized versus cytosolic CaMKII activity can be further controlled by a regulation of dendritic CaMKIIα mRNA transport

and by the regulation of local translation (Mori et al., 2000; Aakalu et al., 2001).

In conclusion, our results demonstrate that CaMKIIβ has a specific role in regulating formation and motility of filopodia as well as synapse formation. This shows that the two major brain isoforms of CaMKII can have fundamentally different and specific functions in neuronal plasticity, and it will be interesting to see how these divergent synaptic strength and morphogenic control mechanisms are implemented in higher brain functions such as learning and memory.

Experimental Procedures

Cell Culture and Transfection

Hippocampal neurons were obtained from 1- to 2-day-old postnatal rats and cultured as described in Shen and Meyer (1999). Neurons were transfected at ages ranging from 1 to 10 days in vitro, and imaged at least 6 hr and in some occasions several days later. Standard transfection protocols for Lipofectamine 2000 were used; each coverslip was treated for 30 min at 37°C with 2 μg of DNA and 3–6 μl of Lipofectamine 2000 (Invitrogen) reagent in osmolarity-corrected OptiMEM (GIBCO) buffer (1 ml transfection volume/coverslip). Neurons were subsequently washed and returned to their original media. For some experiments, transfection was done using a self-built electroporation device (Teruel and Meyer, 1997; Teruel et al., 1999).

RNA Interference

To generate d-siRNA of luciferase and CaMKIIβ, methods were used as described previously (Meyers et al., 2003). A ~600 bp sequence of CaMKIIβ, including the variable region (bp 942–1570 from NCBI Accession Databank number M16112), was used as the template. Neurons were transfected with 25 nM of RNA in the same manner as used for DNA. Peak knockdown of CaMKIIβ was observed after 72 hr (data not shown).

Immunoblotting

Expression analysis by immunoblotting was performed as described previously (Bayer et al., 1998) using specific antibodies against CaMKIIα and β (Cbα2 and Cbβ1; GIBCO) and CaM (Upstate Biotech). Cells from one coverslip (~15–25k neurons) were extracted in 300 μl SDS-loading buffer and 10 μl of the extract was analyzed. Purified protein was used as a standard.

Immunostaining

For immunostaining, cells were fixed in 4% paraformaldehyde for 12 min, permeabilized with 0.1% Triton-X for 10 min, incubated with anti-CaMKIIβ for 1 hr, and incubated with anti-mouse 568 Alexa secondary antibody (Molecular Probes) for 1 hr. All procedures were done at room temperature and washes with PBS/5% FBS were done after each step.

DNA Constructs

Construction and characterization GFP-tagged constructs of wild-type and mutant CaMKII α and β isoforms, CFP, and YFP-Actin have been previous described (Shen et al., 1998, 2000; Shen and Meyer, 1999). To construct the α/β chimera, the variable region of CaMKIIβ (1007–1226 nucleotides) was amplified by PCR and inserted in place of the variable region of CaMKIIα (983–1013 nucleotides). The entire chimera was amplified by PCR and cloned into the Clontech C1 vector. CFP-GPI was generously provided by Dr. P. Keller, PSD95-YFP by Dr. Thierry Galvez, and CFP-Lyn by Dr. Marc Fivaz.

Live Cell Imaging

Dendritic arborization experiments were done at 37°C using a 488 nm excitation laser on a confocal microscope (PASCAL, Zeiss, Inc.). Motility experiments were done at 37°C using 442 nm and 514 nm excitation lasers using a spinning microlens disk confocal microscope (Nipkow Wallac system). The pharmacological agents KN-93, KN-92, calmidazolium, and cytochalasin-D were obtained from Sigma. Peptides were obtained from the Biopolymer Synthesis Center of Caltech. The 27-mer CaM-KIINtide (CNt) is derived from CaM-KIIN and selectively inhibits CaMKII activity (Chang et al., 1998). In order to generate cell-permeable peptides, an antennapedia sequence (RQIKIWFQNRRMKWKK) was placed N-terminal to CNt or a control peptide with reverse sequence (antCNt and antCNt-R); the N-terminal lysine of CNt was substituted by the C-terminal lysine of the antennapedia sequence.

Perimeter Analysis

Using analysis tools in the Metamorph image analysis software, thresholding was first applied to the images of the transfected neuron. In the masked image, the perimeter of the neuron within the field was then calculated. A full field of view was centered on the soma for all measurements (taken with a 63X/1.2 NA Zeiss Planapo objective; zoom 1). The change in branching (%) was calculated relative to GFP controls transfected and imaged on the same day.

Motility Analysis

To determine changes in filopodia and branch motility following an experimental treatment (for instance, KN-93 addition), 2 stacks of 20 images were collected at 20 s intervals (for experiments with mature neurons, 60 s intervals were used) before and after treatment. Metamorph software was used for the analysis. Following photobleaching correction, each stack was duplicated. The last image of the duplicate stack and the first image of the original stack were removed. The original stack was then subtracted from the duplicate stack to obtain a "difference" stack, representing the fluorescence movement over time. The images in the "difference" stack track the movement at each location as a function of time. An image of the integrated local activity was then created by averaging all images in the difference stack. The change in motility (%) was calculated from the mean intensity in this averaged image following drug treatment divided by the averaged intensity before treatment. While spatial differences in fluorescent probe concentration impact the "difference" intensity values, these differences should cancel each other out in the final analysis since all data are presented as "Change in Motility (%)."

F-Actin Binding and CaMKII Activity Assays

Purified actin (from chicken muscle; kindly provided by Dr. R. Rock) was polymerized at a concentration of 4 μM for 5 min on ice in 80 μl F-buffer (25 mM HEPES [pH 7.2], 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, and 0.2 mM ATP). CaMKII and CaM were purified from baculovirus or bacterial expression systems as described previously (Singla et al., 2001). Kinase (100 nM, in 0.1 mg/ml BSA), Ca^{2+} (2 mM) and CaM (1 μM) were added as indicated and incubated for 12 min on ice. To test for association with F-actin, the mixes were spun for 15 min at 100,000 × g; the supernatants and pellets were analyzed for CaMKII content by an overlay assay with biotinylated CaM (STI) as described (Bayer et al., 2001). To test for CaMKII activity, the mix was diluted 1:5 into peptide phosphorylation reactions (25 mM HEPES [pH 7.2], 50 mM KCl, 20 mM $MgCl_2$, 200 μM [γ-^{32}P]ATP at 0.5 Ci mmol-1, and 50 μM AC3 peptide substrate) containing either Ca^{2+}/CaM (2 mM/1 μM; for stimulated activity) or EGTA (1.2 mM; for autonomous activity) and incubated for 1 min at 30°C. The reactions were spotted onto phospho-cellulose paper, the unincorporated radioactivity was removed by a 30–45 min rinse, and ^{32}P incorporation into the peptide substrate was determined in a scintillation counter. To assess phosphorylation of kinase and actin, the reaction mixes (in this case without AC3 substrate) were separated on an SDS-gel and subjected to autoradiography.

Acknowledgments

We would like to acknowledge Dr. Kang Shen for initially observing that CaMKIIβ expression increases dendritic arbor size and for creating several of the mutants used in the study. We would also like to thank Drs. R. Rock and P. Keller for reagents, Drs. Mike Bradshaw and Andy Hudmon for help with CaMKII purification, Drs. Rob Malenka, Stephen Smith, and Cris Neill for comments on the manuscript, and the members of the Meyer lab for general support.

Received: December 23, 2002
Revised: April 8, 2003
Accepted: June 12, 2003
Published: July 30, 2003

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