Activity-Dependent Remodeling of Presynaptic Inputs by Postsynaptic Expression of Activated CaMKII

Kara G. Pratt, Alanna J. Watt, Leslie C. Griffith, Sacha B. Nelson, and Gina G. Turrigiano*
Department of Biology
Volcan National Center for Complex Systems
Brandeis University
Waltham, Massachusetts 02454

Summary

Competitive synaptic remodeling is an important feature of developmental plasticity, but the molecular mechanisms remain largely unknown. Calcium/calmodulin-dependent protein kinase II (CaMKII) can induce postsynaptic changes in synaptic strength. We show that postsynaptic CaMKII also generates structural synaptic rearrangements between cultured cortical neurons. Postsynaptic expression of activated CaMKII (T286D) increased the strength of transmission between pairs of pyramidal neuron by a factor of 4, through a modest increase in quantal amplitude and a larger increase in the number of synaptic contacts. Concurrently, T286D reduced overall excitatory synaptic density and increased the proportion of unconnected pairs. This suggests that connectivity of some synaptic partners was increased while other partners were eliminated. The enhancement of connectivity required activity and NMDA receptor activation, while the elimination did not. These data suggest that postsynaptic activation of CaMKII induces a structural remodeling of presynaptic inputs that favors the retention of active presynaptic partners.

Introduction

During CNS development, the initial patterns of connectivity are highly divergent and must be refined through a process of activity-dependent competition for synaptic space on the postsynaptic neuron (Shatz, 1990; Constantine-Paton and Cline, 1998). For example, early in development LGN neurons receive inputs from >20 retinal ganglion cells, but after a few weeks they are connected to only 1–3 (Sur et al., 1984; Chen and Regehr, 2000). This occurs through a structural remodeling of presynaptic contacts such that some presynaptic partners gain synaptic contacts, while others lose contacts and become entirely disconnected from the postsynaptic neuron. A similar process occurs during the segregation of LGN projections to layer IV of primary visual cortex (Hubel et al., 1977), the reduction in climbing fiber input onto cerebellar Purkinje neurons (Crepel et al., 1978), and loss of multiple innervation at the neuromuscular junction (Purves and Lichtman, 1980; Lichtman and Colman, 2000). These structural rearrangements in presynaptic connectivity depend upon competition between inputs, because activity in one set of inputs is required to drive the elimination of others (Shatz, 1990; Constantine-Paton and Cline, 1998; Lichtman and Colman, 2000). Despite the ubiquity of such structural rearrangements during developmental plasticity, the molecular machinery that allows activity of one presynaptic partner to increase the number of stable synaptic contacts, while eliminating contacts from other inputs, is still unknown.

CaMKII is an attractive candidate to mediate such heterosynaptic structural rearrangements. CaMKII is a serine-threonine kinase that is activated by neuronal activity and can localize to the postsynaptic density (Hanson and Schulman, 1992; Lisman et al., 2002; Leonard et al., 1999; Bayer et al., 2001). Calcium influx through NMDARs allows calcium/calmodulin to bind to CaMKII, which activates the enzyme. Once activated, CaMKII can autophosphorylate on Thr286, which renders the enzyme calcium independent and capable of phosphorylating other substrates even in the absence of calcium. These properties have led to the suggestion that CaMKII is a molecular switch that is moved into a persistently active state by a transient rise in calcium, which can be driven by correlated pre- and postsynaptic activity (Miller and Kennedy, 1986; Ouyang et al., 1997; Lisman et al., 2002).

There is a wealth of evidence that CaMKII is required for activity-dependent synaptic strengthening. CaMKII participates in LTP at central synapses by both phosphorylating synaptic AMPA receptors (AMPARs) and causing insertion of AMPARs into existing synaptic sites (Barria et al., 1997; Lee et al., 2000; Hayashi et al., 2000; Poncer et al., 2002). An additional role in structural plasticity has been suggested by studies showing that CaMKII nulls or transgenes compromise activity-dependent cortical plasticity (Glazewski et al., 1996, 2000; Gordon et al., 1996), although how these effects are mediated at the cellular level remains unknown. The ability of postsynaptic CaMKII to stabilize retinotectal dendrites and axons in Xenopus (Zou and Cline, 1996; Wu and Cline, 1998) and to regulate synaptic density and synaptic structure in invertebrates (Koh et al., 1999; Rongo and Kaplan, 1999) suggests that in addition to postsynaptic changes in receptor trafficking, CaMKII could play an important role in regulating the formation or stability of synaptic connections.

Here we show that postsynaptic activation of CaMKII leads to structural rearrangements that enhance connections from some presynaptic partners, while eliminating connections from others. To directly manipulate the levels of activated CaMKII, we transfected individual cortical pyramidal neurons with a peptide inhibitor of CaMKII, or a constitutively active form of CaMKII (T286D) in which the autophosphorylation at Thr286 was mimicked by changing Thr286 to Asp286. T286D is catalytically active in the absence of calcium and can still form holoenzymes and localize correctly to the PSD (Shen and Meyer, 1999). Expressing T286D in the postsynaptic neuron selectively enhanced connectivity from some presynaptic partners. At the same time, T286D produced a net loss of presynaptic contacts and a reduction in the number of presynaptic partners. The loss of presynaptic
contacts required only constitutive kinase activity, while the enhancement of connectivity required both constitutive kinase activity and ongoing NMDAR activation. These data suggest that postsynaptic activation of CaMKII induces dramatic structural rearrangements in presynaptic connectivity that favors active presynaptic partners.

Results

Experiments were performed on cultured postnatal visual cortical pyramidal neurons between 6 and 8 days in vitro (DIV). These cultures form extensive excitatory and inhibitory networks and develop spontaneous activity after 4–5 DIV (Turrigiano et al., 1998). In these postnatal cultures, many excitatory neurons retain a pyramidal morphology with a prominent apical-like dendrite, and so can be identified morphologically (Watt et al., 2000; Kilman et al., 2002). To examine the downstream effects of postsynaptic CaMKII activation on synaptic connectivity, we directly increased the amount of activated CaMKII in individual pyramidal neurons. This was accomplished by cotransfecting with EGFP and a mutated α-CaMKII in which autophosphorylation at T286 was mimicked by changing T286 to an aspartate (T286D) (Figure 1A; Waldmann et al., 1990). Neurons were transfected for 16–20 hr at low efficiency (1–6 neurons/dish) (Kilman et al., 2002). This allowed us to alter CaMKII signaling in individual postsynaptic neurons without directly affecting CaMKII signaling in presynaptic partners. This selectivity is important because CaMKII is present at high levels in presynaptic terminals (Lui and Jones, 1996), so altering pre- and postsynaptic signaling together could have confounding effects on synaptic transmission and connectivity. Expression of a T286D-GFP fusion protein demonstrated low-level diffuse dendritic expression and enrichment at synaptic sites (Figures 1B and 1C), a distribution similar to that seen for endogenous CaMKII (see Figure 7A). Expression of fluorescent protein was evident within 3–6 hr of transfection and was stable for several days.

Constitutively Active CaMKII Dramatically Increased the Strength of Paired Transmission

To measure the strength of evoked transmission, we obtained recordings between monosynaptically coupled pairs of pyramidal neurons, in which the presynaptic neuron was untransfected and the postsynaptic neuron was either untransfected (Figure 2A, Control) or was cotransfected with EGFP and T286D (Figure 2A, T286D). On average, postsynaptic T286D increased EPSC amplitude between connected pairs by 3.9-fold, from $-71 \pm 19$ pA to $-278 \pm 45$ pA (Figures 2A and 2B, left; T286D significantly different than control, t test, p < 0.0002, n = 10 and 13 pairs, respectively). There were no significant differences in EPSC rise or decay times between conditions (rise = $1.4 \pm 0.1$ and $1.5 \pm 0.1$ ms; decay = $8.0 \pm 1.6$ and $10.1 \pm 2.7$ ms, for control and T286D pairs, respectively). Transfection with EGFP and a T286A mutant kinase, which cannot autophosphorylate on residue T286 and so cannot become calcium independent, did not increase evoked transmission (Figure 2B, n = 3). For these experiments and those described below, transfection with EGFP alone had no effect on synaptic transmission or passive neuronal properties; in addition, none of the mutant kinases had significant effects on passive cell properties or on the kinetics of synaptic currents.

A change in evoked transmission could arise from postsynaptic changes in the amplitude of quantal currents, due to insertion or phosphorylation of synaptic AMPARs. Previous studies have reported that the catalytic domain of CaMKII can cause insertion of AMPARs into the postsynaptic membrane (Hayashi et al., 2000; Poncer et al., 2002). Consistent with this, we found that transfection with T286D for 16–20 hr shifted the distribution of AMPA-mediated miniature excitatory postsynap-
Structural Synaptic Plasticity Induced by CaMKII
271

Figure 2. Increasing the Amount of Activated CaMKII in the Postsynaptic Neuron Dramatically Increased Quantal Content
(A) Representative paired recordings between a pair of control neurons or a pair where the postsynaptic neuron expressed EGFP and T286D. Thin gray lines represent individual trials; thick lines, the average EPSC.
(B) Average EPSC amplitude (left axis) for the indicated condition (n = 13 control, 10 T286D, and 3 T286A pairs; asterisk indicates T286D significantly different from control, p < 0.001, corrected t test). Average mEPSC amplitude (right axis) for the indicated condition (n = 12 control, 8 T286D, and 5 T286A neurons, asterisk indicates T286D significantly different from control, p < 0.02, corrected t test).
(C) Amplitude distribution of mEPSCs from control neurons (n = 12) or neurons transfected with T286D (n = 8). Insets show the average mEPSC for the indicated condition. Scale bar equals 10 pA (vertical) and 10 ms (horizontal).
(D) Both the average quantal content (average EPSC amplitude/average mEPSC amplitude) and 1/CV of EPSC amplitude were increased more than 2-fold by T286D expression. Asterisk indicates T286D significantly different from control, p < 0.02).

T286D also increases either the probability of transmitter release (P) or the number of functional release sites (N) that mediate connectivity onto T286D-expressing neurons. Consistent with this, T286D significantly reduced the coefficient of variation (CV) of EPSC amplitude, from 0.36 ± 0.04 to 0.23 ± 0.03 (p < 0.03), as predicted for a change in either P, or N (Faber and Korn, 1991; Korn and Faber, 1991). Re-expressing this as 1/CV² (which is directly proportional to N; Faber and Korn, 1991) demonstrated that T286D expression increased this quantity 2.3-fold (Figure 2D, T286D significantly different from control, p < 0.02).

T286D Does Not Alter Short-Term Plasticity
A change in release probability is predicted to alter the short-term plasticity of synaptic transmission (Zucker and Regehr, 2002). If the increase in quantal content were due to increased release, short-term depression (STD) would be expected to increase. To determine...
T286D Increases the Number of Synaptic Contacts Mediating Paired Transmission

The lack of effect of T286D on short-term plasticity suggests that the increase in quantal content is primarily due to an increase in the number of functional release sites mediating transmission between synaptically coupled partners. This could be due either to growth of new connections or from conversion of existing nonfunctional contacts into functional ones. One mechanism to increase the number of functional excitatory contacts is to decrease the proportion of “silent” synapses—excitatory presynaptic contacts that are not apposed to functional AMPAR clusters (Liao et al., 1995; Isaac et al., 1997). This possibility is unlikely because the proportion of silent synapses is quite low in these postnatal cortical cultures. NMDA-only synapses between visual cortical neurons decline developmentally and are largely gone by postnatal day 8–10 (Isaac et al., 1997; Rumpel et al., 1998). In our postnatal cultures, colocalization of synapsin I and AMPAR using an antibody directed against an extracellular epitope of GluR1 revealed that 68% ± 9% of synapsin puncta were colocalized with AMPAR (n = 7 neurons). As 25%–30% of synapsin puncta onto these neurons arise from inhibitory presynaptic terminals (Kilman et al., 2002), at most 10% of excitatory presynaptic terminals fail to cluster AMPARs under control conditions. This indicates that conversion of existing AMPAR-lacking synapses into AMPAR-containing synapses could account for only a small fraction of the increased EPSC amplitude we observe following T286D expression.

Next, we asked whether T286D alters the number of physical synaptic contacts mediating excitatory transmission. To measure the average number of synaptic contacts between pairs of pyramidal neurons, we transfected cultures with EGFP or EGFP + T286D and then filled a nearby pyramidal neuron (somata within 700 μm) with biocytin, which was visualized with Texas red-conjugated avidin. Cultures were then fixed and stained against synapsin, and the total number of putative synaptic contacts (Np) from one presynaptic axon onto the dendrites of one postsynaptic EGFR or EGFP + T286D expressing neuron was quantified. Synaptic contacts were defined as sites of axo-dendritic contact where the presynaptic axon clustered synapsin; typically these sites had morphological specializations such as dendritic protrusions or filopodia and presynaptic swellings (Figure 4A, inset). Over the spatial scale we examined (somas < 700 μm apart), there was no correlation between Np and the distance between partners (p > 0.5).

In some cases, axons of filled neurons contacted the dendritic arbor of transfected neurons, often multiple times, without forming presynaptic contacts that clustered synapsin. Such pairs (Zeros) were infrequent when the postsynaptic neuron expressed EGFP (10% of cases, Figure 4B, top panel), but the frequency increased 4-fold when the postsynaptic neuron expressed T286D (42%, Figure 4B, bottom panel), indicating that T286D-expressing neurons receive synaptic contacts from fewer of their potential presynaptic partners. For control neurons, Np was unimodally distributed, while for T286D-expressing neurons the distribution of Np was bimodal, with a large peak at 0 and a second peak

---

**Figure 3.** Activated CaMKII in the Postsynaptic Neuron Did Not Significantly Affect the Short-Term Plasticity of Synaptic Transmission (A) Representative recordings from a control pair or a pair in which the postsynaptic neuron expressed EGFP and T286D, showing the response to a 20 Hz train of action potentials. Synaptic current traces are the average of >10 trials. (B) Average depression (amplitude of last response/amplitude of first response) for control or postsynaptic-T286D pairs at 5, 20, and 40 Hz; differences were not significant (ANOVA > 0.23, n = 7 control and 6 T286D pairs).
Figure 4. Activated CaMKII in the Postsynaptic Neuron Increased the Number of Synaptic Contacts Mediating Synaptic Transmission

(A) A postsynaptic neuron transfected with T286D/EGFP (blue), contacted by a presynaptic neuron visualized with biocytin (red); scale bar equals 50 μm. Inset: a region of axon-dendrite contact from the area indicated by box. Clockwise from top right: synapsin (green), dendrite (blue), and axon (red) from the same field of view, and an overlay of all three in the top left panel. Scale bar equals 3 μm.

(B) Histogram of the number of synaptic contacts between pairs for control (top, n = 10) or pairs where the postsynaptic neuron expressed T286D (bottom, n = 19 pairs). Zeros indicate pairs with no detectable synapses despite having regions of axon-dendrite contact. Average number of contacts (excluding Zeros) was increased 2.2-fold by T286D (significant, p < 0.001).

centered around 20. Excluding the Zeros, the mean N_p for control pairs was 8.2 ± 1.7, while for T286D pairs it was 18.6 ± 1.6. This 2.2-fold change in N_p is similar to the 2.3-fold change in 1/CV^2 of EPSC amplitude (Figure 2D) and can account for much of the estimated 2.8-fold change in average quantal content produced by T286D.

It is worth noting that the EPSC amplitude measurements are derived only from physiologically connected pairs, which would correspond to the anatomically defined pairs with non-zero values of N_p. The bimodal distribution of N_p onto T286D-expressing neurons suggests that T286D induced a redistribution of synaptic contacts...
so that, on average, one synapse is formed for every two axon-dendrite crossings, regardless of condition.

We calculated separately the average number of axon-dendrite crossings for control and T286D pairs that formed synaptic contacts, and for Zeros (pairs with axon-dendrite contacts but no synapses). The number of axon-dendrite contacts varied significantly with condition, being higher in T286D pairs than in Zeros or Controls (Figure 5B, left panel; ANOVA, $p < 0.003$; T286D significantly different from Control and Zeros, $p < 0.03$ and 0.008, respectively). T286D produced a similar trend in the total length distance that axons remained in contact with the postsynaptic dendrite (Figure 5B, right panel, ANOVA, $p < 0.003$; Zeros significantly different from Control and T286D, $p < 0.03$ and 0.003, respectively).

Quantification of the apical dendritic structure of control and T286D expressing neurons (Figure 5C, 6–8 neurons/condition) revealed no significant differences in total apical dendritic branch length (TADBL), branch number (Br #), or in the length density of dendritic filopodia (defined as terminal branches < 10 μm in length). In addition, no significant effects on branch order were found (data not shown; ANOVA, $p < 0.99$). The effects of T286D on axo-dendritic crossings and synapse number are therefore not due to a net increase in dendritic branching. Taken together, these results suggest that postsynaptic T286D influences connectivity either by directly influencing the axonal branching of presynaptic partners or by influencing the number of axo-dendritic contacts that are stabilized.

**T286D Decreases the Average Density of Excitatory Synapses**

The 4-fold increase in the proportion of pyramidal neuron pairs that did not form synaptic contacts suggests that T286D may cause a net loss of excitatory synapses. To examine this possibility, we quantified the average length density (number of synapses/unit length of dendrite) of synaptic contacts onto the apical-like dendrites of transfected pyramidal neurons. Cotransfection with EGFP allowed the entire dendritic arbor of individual neurons to be visualized (Figure 1A). Neurons were then fixed and stained for the presynaptic marker synapsin (Figure 6A) and/or the postsynaptic marker of excitatory synapses, PSD-95 (Figure 6C), and the number of puncta/unit length of EGFP-filled dendrite was quantified and compared to that of neurons transfected with EGFP alone. For the immunohistochemical experiments described here and below, each experiment represents data from an independent plating in which sister cultures were transfected with the fluorescent protein ± the indicated construct, and were fixed, stained, and quantified in parallel. For each experiment, 2–8 neurons/condition were quantified and the data averaged.

Transfection with T286D for 16–20 hr produced on average a 35% ± 4% reduction in the density of synaptical puncta (Figures 6B and 6D; T286D versus EGFP, $p < 0.002$, $n = 8$ experiments). Over this same 16–20 hr culture period, control synaptic density did not increase significantly (6.3% ± 9.2%, $n = 3$ experiments), so this reduction represents a net loss of about 25%–30% of presynaptic contacts during the period of transfection.
This could occur through a reduced rate of synapse addition, or an increased rate of synapse loss due to failure to stabilize contacts once they are formed. Transfection with the T286A mutant had no significant effect on synapse density (118% ± 15% of control, n = 3 experiments, p > 0.78). To verify that the synapse loss induced by T286D required phosphorylation of substrates by CaMKII, we transfected neurons with the double CaMKII mutant K42M/T286D, which cannot hydrolyze ATP and so cannot phosphorylate substrates despite being in the active conformation (Hanson et al., 1994). Mutation of the ATP binding site prevented the synapse elimination produced by T286D and in fact increased synaptic density to 148% ± 21% of control values (n = 4 experiments), suggesting that K42M/T286D is acting as a dominant negative. Taken together, these data indicate that the net loss of presynaptic contacts induced by CaMKII requires both constitutive kinase activity and the ability to phosphorylate substrates.

To determine whether T286D was targeting excitatory synapses, triple-label experiments were performed so we could simultaneously localize presynaptic (synapsin, in green pseudocolor) and excitatory postsynaptic (PSD-95, in red pseudocolor) structures onto EGFP-filled dendrites (in blue pseudocolor, Figure 6C). Approximately 72% of PSD-95 puncta were colocalized with synapsin, and the degree of colocalization was not affected by T286D (T286D was 99.9% ± 7.0% of control, n = 4 experiments). T286D expression reduced the density of PSD-95 (PSD) puncta, and the density of puncta where the two markers were colocalized (PSD/Syn), to a similar degree (by approximately 33%, Figure 6D, n = 4 experiments, T286D significantly different from EGFP alone, p < 0.01), indicating that the reduction in presynaptic contacts can be accounted for by loss of excitatory terminals.

Some immature presynaptic contacts may not be capable of transmitter release. To determine whether T286D reduced the density of functional presynaptic contacts, we used the steryl dye FM1-43 to measure the density of presynaptic contacts that were capable of releasing and recycling synaptic vesicles. Postsynaptic dendrites were visualized by transfection with the red fluorescent protein DsRed, and FM1-43 was loaded using a standard protocol (Kavalali et al., 1999; Murthy et al., 1997). The range of densities of presynaptic contacts measured with FM1-43 (1.8 ± 0.3 puncta/10 μm dendrite) was comparable to the average density measured (in separate experiments) with synapsin (1.5 ± 0.3 puncta/10 μm dendrite), suggesting that the majority of presynaptic contacts were capable of vesicle release and recycling. T286D produced a 33% reduction in FM1-43 puncta, comparable to the reduction in synapsin puncta (Figure 6D, FM1-43; T286D significantly different from DsRed alone, p < 0.002, n = 4 experiments).

This reduction in excitatory synapse density occurred without any dramatic changes in dendritic morphology (Figure 5C). Taken together, these data indicate that increasing the amount of constitutive CaMKII activity in the postsynaptic neuron causes a net loss of excitatory
synaptic contacts. These data are consistent with previous reports of the effects of overexpressing wild-type αCaMKII, which decreases mEPSC frequency and increases mEPSC amplitude in cultured hippocampal neurons (Thiagarajan et al., 2002).

Inhibition of Endogenous CaMKII Increases Synaptic Density and Decreases mEPSC Amplitude

In rodent visual cortex, endogenous CaMKII levels increase steeply during the first few weeks of postnatal development (Sahyoun et al., 1985). There are detectable levels of endogenous CaMKII present after 6–7 DIV (equivalent to about postnatal day 10–11; Figure 7A), prompting us to examine the role of this endogenous kinase in regulating synapse density onto pyramidal neurons. To accomplish this, we coexpressed EGFP and the autoinhibitory peptide ala, which binds to endogenous CaMKII and prevents its activation (Griffith et al., 1993). While increasing the amount of constitutive kinase activity with T286D decreased synapse density (Figures 6B and 6D), inhibition of endogenous kinase with ala peptide for 16–24 hr increased synapse density to 120.1% ± 6.0% of control values (Figures 7B and 7D; ala versus EGFP alone, p < 0.02, n = 5 experiments).

These data show that synapse density can be bidirectionally regulated by the amount of activated CaMKII in the postsynaptic neuron.

Inhibition of endogenous CaMKII with ala peptide also affected mEPSC amplitude. Transfection with ala decreased mEPSC amplitude to 77.9% ± 5.3% of control values (Figures 7C and 7D). Manipulating the level of activated postsynaptic CaMKII therefore had opposite effects on the number and unit strength of excitatory synaptic contacts. T286D reduced the number of functional synaptic contacts but increased the quantal amplitude of those that remained, while blocking activation of endogenous CaMKII with α peptide increased the number of contacts but reduced their strength.

T286D Increases Connectivity in an Activity-Dependent Manner

Activated CaMKII has two seemingly paradoxical effects on synapse density. The overall density of excitatory contacts is decreased by T286D expression, as is the number of synaptic partners, suggesting that some presynaptic partners are eliminated. At the same time, the number of contacts between partners that remain connected increases. Because T286D is expressed throughout the postsynaptic neuron, it is not clear why some
Structural Synaptic Plasticity Induced by CaMKII

Figure 8. The Enhancement of Transmission Induced by Activated CaMKII Is Activity Dependent, while the Synapse Loss Is Not

(A) Average density of synapsin puncta onto T286D-expressing neurons grown with or without TTX in the medium during the period of transfection, expressed as a percent of control. T286D not significantly different from T286D + TTX (n = 4 experiments).

(B) EPSC amplitude from pairs in which the postsynaptic neuron expressed T286D, grown with or without TTX in the medium during the period of transfection. Data are expressed as percent of control EPSC amplitude (n = 10 and 5 pairs, respectively, for T286D and T286D + TTX).

(C) Coefficient of variation (CV) for EPSC amplitudes in (B). For (B) and (C), T286D significantly different from control (p < 0.001 and p < 0.03, respectively, corrected t test; T286D + TTX not significantly different from control).

(D) Number of synaptic connections (excluding Zeros) between pairs of neurons, expressed as % of control. The increase in Nc produced by postsynaptic T286D was prevented by either TTX or NMDAR blockade during the period of transfection (T286D significantly different from control, p < 0.001, and significantly different from T286D + activity block, p < 0.02).

In contrast, the increase in paired transmission produced by T286D was completely blocked by including TTX in the medium during the period of transfection (Figure 8B). In order to avoid inducing a homeostatic upregulation of EPSC amplitude from the activity blockade (Turrigiano et al., 1998), we minimized the period of TTX treatment by adding TTX 3 hr after transfection when T286D expression is just coming on. TTX alone for this period of time (approximately 13 hr) did not increase EPSC amplitude (TTX was 83% ± 13% of control, n = 4 pairs), as shown previously (Turrigiano et al., 1998). In addition to blocking the effects of T286D on EPSC amplitude, TTX also prevented the change in the CV of the EPSC amplitude (Figure 8C).

In keeping with the ability of TTX to block the increase in EPSC amplitude produced by T286D, TTX also blocked the increase in Nc (excluding Zeros) induced by T286D (Nc for TTX + T286D was 9.7 ± 5.6, n = 5 pairs; significantly different from T286D alone, p < 0.02, and not significantly different from control, p > 0.98). We wondered whether complete activity blockade was necessary to prevent the increase in Nc, or whether blocking NMDAR was sufficient. To test this, cultures were incubated in APV and MK801 during the period of transfection, and the average number of synaptic contacts between connected pairs was analyzed. NMDAR blockade completely prevented the increase in Nc produced by T286D (n = 9 pairs, T286D + NMDAR block significantly different from T286D alone, p < 0.001; values for T286D + TTX and T286D + NMDAR block were not significantly different, so data were combined, Figure 8D). These data indicate that constitutive CaMKII activity alone is not sufficient to induce the dramatic potentiation of paired transmission, but that activation of NMDAR by ongoing activity is also required.

Discussion

Activity-dependent developmental plasticity is ultimately expressed through structural changes in synaptic connectivity (Shatz, 1990). Similarly, while the initial stages of memory formation probably involve alterations in the strength of existing synapses (Lisman et al., 2002), long-term memory storage is thought to require a structural remodeling of presynaptic connectivity (Bailey and Kandel, 1993). Our data demonstrate that in addition to strengthening synaptic transmission through postsynaptic changes in AMPAR function, constitutive CaMKII activity in the postsynaptic neuron causes an increase in the number of synaptic contacts mediating transmission from presynaptic partners. This structural enhancement of connectivity requires the conjunction of activated postsynaptic kinase and ongoing NMDAR activation.

At the same time that some presynaptic partners gain...
connections onto the postsynaptic neuron in an activity-dependent manner, our data suggest that connections from other partners are eliminated altogether. Unlike the enhancement of connectivity, this elimination does not require ongoing activity in the presence of activated kinase. Once correlated pre- and postsynaptic activity activates CaMKII in the postsynaptic neuron, it can therefore either enhance or decrease connectivity from presynaptic partners. Our data suggest that the inputs that are structurally enhanced are those that are effective at continuing to drive the postsynaptic neuron and activate NMDAR.

CaMKII has long been known to increase the strength of synaptic transmission through postsynaptic mechanisms. CaMKII can directly insert AMPARs into the postsynaptic membrane (Hayashi et al., 2000), can phosphorylate AMPARs and increase their conductance (Barria et al., 1997; Lee et al., 2000), and can also cause a decrease in the proportion of existing synaptic sites that do not express functional AMPARs (Poncer et al., 2002). Consistent with these previous reports, we find that increasing the amount of constitutive CaMKII activity in cortical pyramidal neurons induces modest but significant changes in AMPA quantal amplitude. However, we also find a dramatic increase in the quantal content of paired synaptic transmission that can largely be accounted for by a 2.2-fold increase in the number of synaptic contacts arising from presynaptic partners.

One issue raised by these results is what proportion of these morphologically identified sites of synaptic contact represents functional synapses (Ahmari and Smith, 2002). Several lines of evidence suggest that the majority of such sites do represent functional synapses. (1) The density of synapsin and FM1-43 puncta corresponds well, suggesting that most or all synapsin puncta represent functional presynaptic release sites. A similar correspondence between FM1-43 labeling and presynaptic markers has been reported previously (Sporns and Jenkinson, 1995; Hof et al., 2002). (2) The expected proportion (approximately 70%) of synapsin puncta is colocalized with the excitatory synaptic markers PSD-95 and GluR1. Previously we have shown that the remaining 25%–30% of presynaptic contacts are inhibitory (Kilman et al., 2002). (3) Finally, the morphological change in connectivity induced by T286D (2.2-fold) is on the same order as the change in 1/CV² of EPSC amplitude (2.3-fold), as predicted if the change in CV is due to a change in the number of functional release sites (Faber and Korn, 1991).

The changes in presynaptic connectivity induced by T286D are bimodal. Presynaptic partners that remain connected gain synaptic contacts. At the same time, there is a dramatic increase in the proportion of potential synaptic partners that do not form synaptic connections with the postsynaptic neuron, and this is coupled to a net loss of excitatory synapses during the transfection period. Taken together, these data strongly suggest that T286D is selectively eliminating synaptic connections from some presynaptic partners. While a role for CaMKII in synapse elimination has not previously been described in vertebrate neurons, a similar ability of constitutively active CaMKII to lower excitatory synapse density has been described in C. elegans (Rongo and Kaplan, 1999).

In central neurons (Liu and Tsien, 1995; Turrigiano et al., 1998; Desai et al., 2002) and at the fly neuromuscular junction (Davis and Goodman, 1998), it has been suggested that there is a homeostatic trade-off between synapse number and synaptic strength. Increased excitatory synapse number, by increasing the activity of the postsynaptic neuron, could drive an activity-dependent reduction in quantal amplitude (Turrigiano et al., 1998; Desai et al., 2002). Our data do not rule out the possibility that changes in quantal amplitude induced by T286D and ala peptide are an indirect consequence of changes in synaptic density, but the ability of truncated CaMKII to rapidly induce changes in quantal amplitude suggest that this is unlikely (Pettit et al., 1994; Hayashi et al., 2000). Conversely, the net loss of synaptic contacts induced by T286D could be an indirect effect of synaptic potentiation, mediated through an increase in neuronal activity. This does not appear to be the case as T286D induces synapse loss even when activity is blocked, indicating that the loss is independent of any changes in neuronal activity. These considerations suggest that CaMKII might produce in neuronal activity. These considerations suggest that CaMKII has independent (and opposing) effects on AMPA quantal amplitude and the density of excitatory synapses.

We have found that excitatory synapse density can be bidirectionally regulated by increasing or decreasing postsynaptic CaMKII signaling. The degree of activated CaMKII in the postsynaptic neuron may therefore act as a limiting factor during synaptogenesis, so that as CaMKII activity rises developmentally (Sahyoun et al., 1985; Zou and Cline, 1996), synaptogenesis is slowed. A role for CaMKII in limiting synaptic density accords well with several previous findings. First, postsynaptic expression of constitutively active CaMKII stabilizes both dendritic arbors and presynaptic axon terminals (Zou and Cline, 1996; Wu and Cline, 1998), which could reduce synaptogenesis by slowing the rate at which new contacts are formed. Second, it has been observed that blocking NMDA receptor signaling enhances excitatory synaptogenesis in hippocampal slice cultures (Luthi et al., 2001), suggesting that synapses are normally pruned through an NMDA receptor-dependent mechanism. Our data suggest that this NMDA receptor-dependent pruning could be mediated through CaMKII activation. A limiting role for CaMKII in synaptogenesis raises the interesting possibility that if CaMKII activation were to fall late in development (due, for example, to lowered activity following denervation or other pathological circumstances), this might have the consequence of once again enhancing synaptogenesis.

Consistent with the in vitro ability of CaMKII to induce structural synaptic rearrangements demonstrated here, autophosphorylation of CaMKII plays an important role in competitive, experience-dependent plasticity in vivo. Both ocular dominance (OD) plasticity and whisker deprivation (WD) plasticity are impaired in transgenic mice possessing the T286A point mutation inCaMKII, indicating that autophosphorylation is necessary for these forms of cortical plasticity (Glazewski et al., 2000; Taha et al., 2002). The effects of constitutively active CaMKII on sensory responses and on WD plasticity are more complex and depend both on the age of expression and on expression levels (Glazewski et al., 2001). When the transgene is expressed early in development, whisker
responses are less selective (consistent with a generalized potentiation of inputs), but WD plasticity is still present. Some of this complexity probably arises because CaMKII activity is elevated for a prolonged period of time (weeks), both pre- and postsynaptically, and throughout the cortical layers in which it is normally expressed. While it is difficult to extract from these in vivo studies the cellular mechanisms by which CaMKII mutations influence cortical connectivity, they are in general consistent with the idea that CaMKII plays a role in experience-dependent synaptic rearrangements.

Wild-type CaMKII is moved into a persistently active state in an activity- and NMDAR-dependent manner (Ouyang et al., 1997). Once CaMKII has been activated, it can induce synapse loss with no additional requirement for ongoing activity. In contrast, our data suggest that activation of CaMKII is not sufficient to generate structural enhancement of connectivity but must occur in conjunction with ongoing electrical activity and NMDAR activation. Functionally, this requirement for NMDAR activation suggests that only inputs that are active in the presence of constitutively active CaMKII will be enhanced, while inactive inputs will be eliminated. This requirement for NMDAR activation in the presence of activated CaMKII may guarantee that only those inputs that persistently activate the postsynaptic neuron undergo structural enhancement.

Many structural rearrangements in connectivity are competitive, in that patterns of activity that increase connectivity from some synaptic partners also lead to loss of inputs from others (Hubel et al., 1977; Chen and Regehr, 2000; Lichtman and Colman, 2000). Synaptic competition at the NMJ is thought to require an unidentified heterosynaptic “punishment” signal that is generated in the postsynaptic neuron by correlated inputs, and then is able to cause elimination of other, uncorrelated, inputs (Lichtman and Colman, 2000). Similarly, it has been postulated that in central circuits, activation of NMDA receptors generates a heterosynaptic signal that eliminates inactive inputs (Constantine-Paton and Cline, 1998). Our data suggest that inputs that are effective at activating CaMKII in the postsynaptic neuron (and remain active) will simultaneously enhance themselves and bias inactive synapses toward elimination. The ability of CaMKII to differentiate between active and inactive synapses may allow the activity-dependent activation of a single molecular signal to generate competitive structural rearrangements in synaptic connectivity.

**Experimental Procedures**

Experiments were performed on visual cortical cultures derived from p3-5 Long-Evans rat pups as previously described (Turrigiano et al., 1998; Watt et al., 2000), except neurons were plated onto astrocyte beds and medium was supplemented with B27 (Life Sciences). All experiments were performed in parallel on control and experimental neurons or pairs from the same platings; data are expressed as mean ± SEM. Transfections were performed after 6 days in vitro using a Bio-Rad gene gun as described (Kilman et al., 2002); for cotransfections, mammalian expression plasmids containing the genes of interest were mixed prior to precipitation onto the gold particles. Synaptic parameters were analyzed 18–20 hr after transfection. Over this period of time in culture, neither control EPSC amplitude (97% ± 26% of control, n = 6 in each condition) nor control synapse density (106% ± 9% of control) changed significantly.

MEPSCs and paired recordings between pyramidal neurons were obtained and quantified as previously described (Turrigiano et al., 1998; Watt et al., 2000); ANOVAs followed by unpaired two-tailed t tests corrected for multiple comparisons were used to determine statistical significance. To record from transfected neurons, EGFP-expressing neurons were identified using fluorescence, and recordings were then obtained after switching to DIC. Recordings were discarded if resting potentials were >–55 mV, input resistances were <–200 MΩ, or either of these parameters changed by more than 10% during the course of the recording.

For quantifying synapse densities and synaptic protein distributions, neurons were fixed, imaged using an Olympus IX70 inverted microscope with a 60× objective (NA 1.25), and photographed using a cooled CCD camera (Sensys, Photometrics) as described (Kilman et al., 2002). All data collection and analysis were performed blind to experimental condition. Biocytin fills and synaptic markers were visualized with Texas red or Cascade blue secondary antibodies; by switching between filters, the same field of view was captured for the different fluorophores; images are presented in pseudocolor. To quantify the number of connections between pairs of neurons (Np), all regions of axo-dendritic contact were photographed in the red (axon), green (dendrite), and blue (synapsin) channels. Neutral density filters were used to reduce bleaching. Putative synapses were defined as axo-dendrite contacts that colocalized synapsin; these contacts typically had axonal swellings and dendritic swellings or filopodia, while regions of axo-dendritic contact that did not accumulate synapsin were absent such specializations. To load presynaptic sites with FM1–43 (Molecular Probes), cultures were washed into 0 Ca2+ (ACSF containing 0.1 μM TTX, 50 μM APV, and 20 μM CNQX), then perfused for 120 s with a depolarizing solution containing 50 mM KCl, 2.5 mM CaCl2, and 10 μM FM1–43, and washed back into 0 Ca2+ ACSF for at least 20 min prior to data collection. Opentab software (improvement) was used to quantify the number of fluorescent puncta/unit length of EGFP or DsRed-filled dendrite; puncta that were overlapping or contiguous with filled dendrites were counted.

For synapse density experiments, 2–8 neurons/condition were quantified and values averaged for the number of independent experiments (from separate platings) indicated; paired t tests were used to determine statistical significance. Initially all presynaptic contacts onto the entire apical-like dendrite were quantified; similar results were obtained by counting the distal apical branches only (where the majority of synaptic contacts were made), so in subsequent experiments this method was used. Using the latter method, several hundred microns of distal apical dendrite per neuron were quantified.

**Acknowledgments**

T286D, T286A, and K42M/T286D mutant kinases were the kind gift of Dr. Howard Schulman. We thank Mark Mattaliano for construction of the GFP-CaMKII fusion vector, Per Jesper Sjöström for help with analysis software, and Tanya Casimiro and Valerie Kilman for technical assistance. This work was supported by NIH NS36855 and NSF IBN 9726944.

Received: October 18, 2002
Revised: June 18, 2003
Accepted: June 25, 2003
Published: July 16, 2003

**References**


Bayer, K.-U., De Koninck, P., Leonard, A.S., Hell, J.W., and Schul-


Structural Synaptic Plasticity Induced by CaMKII


