Calcium Regulation of Dendritic Growth via CaM Kinase IV and CREB-Mediated Transcription

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Summary

We report that CaM kinase IV and CREB play a critical role in mediating calcium-induced dendritic growth in cortical neurons. Calcium-dependent dendritic growth is suppressed by CaM kinase inhibitors, a constitutively active form of CaM kinase IV induces dendritic growth in the absence of extracellular stimulation, and a kinase-dead form of CaM kinase IV suppresses dendritic growth induced by calcium influx. CaM kinase IV activates the transcription factor CREB, and expression of a dominant negative form of CREB blocks calcium- and CaM kinase IV-induced dendritic growth. In cortical slice cultures, dendritic growth is attenuated by inhibitors of voltage-sensitive calcium channels and by dominant negative CREB. These experiments indicate that calcium-induced dendritic growth is regulated by activation of a transcriptional program that involves CaM kinase IV and CREB-mediated signaling to the nucleus.

Introduction

Neuronal activity exerts a profound influence on the patterning of dendrites during development (reviewed in Cline, 2001). This view is based on an extensive literature of activity deprivation experiments, which suggest that loss of normal activity during development leads to lasting deficits in dendritic development. For example, activity blockade by various pharmacological interventions leads to deficits in dendritic growth in Xenopus tectal neurons (Rajan and Cline, 1998), and in the mammalian visual system, monocular deprivation alters dendritic development in the lateral geniculate nucleus and the visual cortex (Coleman and Riesen, 1968; Wiesel and Hubel, 1963). A role for neuronal activity in the development of visual cortical neurons is also supported by the observation that exposure to enriched environments increases dendritic branching in cortical pyramidal neurons and that elaboration of dendrites in cortical slices can be attenuated by activity blockers (Holloway, 1966; Greenough and Volkmar, 1973; Volkmar and Greenough, 1972; Juraska, 1982; McAllister et al., 1996). Neuronal activity has also been implicated in the control of dendritic development of auditory brainstem neurons, cerebellar Purkinje cells, spinal motor neurons, and cilliary ganglia (Rakic and Sidman, 1973; Sotelo, 1975; Mason et al., 1997; Benes et al., 1977; Deitch and Rubel, 1984; Purves and Hume, 1981; Kalb, 1994).

Much of our understanding of the relationship between neuronal activity and dendritic development has come from studies in the Xenopus retinotectal system where timelapse imaging has allowed detailed investigations of dendritic development in vivo. In Xenopus, the onset of dendritic development closely parallels the formation of the first synaptic inputs (Wu et al., 1996). The dendritic arbor rapidly expands over the subsequent few days (Wu et al., 1999). During this period of rapid dendritic growth, synaptic currents are principally mediated by NMDA receptors, and pharmacological blockade of NMDA receptors markedly reduces dendritic growth rates (Rajan and Cline, 1998).

While these observations indicate that afferent activity plays a critical role in regulating dendritic growth during development, the underlying molecular mechanisms have not yet been identified. For example, it is not known if neuronal activity influences dendritic growth by inducing local cytoskeletal changes at sites of synaptic contact, or if activity-dependent dendritic growth requires signaling to the nucleus. Since the long-term biochemical and cellular effects of neuronal activity are mediated by calcium influx (Ghosh and Greenberg, 1995), it is likely that the effects of activity on dendritic growth are mediated by calcium signaling. Here we report on our investigations of the signaling mechanisms by which calcium influx regulates dendritic growth in cortical neurons.

Results

The effects of neuronal activity on dendritic growth were examined in cortical cultures prepared from embryonic day 18 rats. The dendritic morphology of individual neurons was visualized by transfection of β-galactosidase at E18 + 2 days in vitro (DIV) followed by β-galactosidase immunocytochemistry 2 days post-transfection. This method reveals the detailed dendritic morphology of transfected neurons and can be used to evaluate the role of different signaling pathways in regulating dendritic growth (Threadgill et al., 1997). As shown in Figures 1E and 1G, cortical neurons in culture elaborate well-differentiated dendritic trees by 4 days in culture, which allows for quantitative analysis of dendritic parameters.

Extracellular calcium enters neurons primarily via NMDA receptors and voltage-sensitive calcium channels (VSCCs) (Ghosh and Greenberg, 1995). To determine if calcium influx via these channels contributed to dendritic growth under basal conditions, we examined the consequences of treating the cultures with antagonists of the NMDA receptor or VSCCs. While treatment of the cultures with the NMDA receptor antagonist APV had no detectable effect on dendritic growth, inhibition of L-type VSCCs (L-VSCCs) with Nifedipine resulted in a significant reduction in dendritic growth (Figures 1A and 1B). Thus calcium influx via VSCCs, but not NMDA receptors, influences dendritic growth under basal activity conditions.

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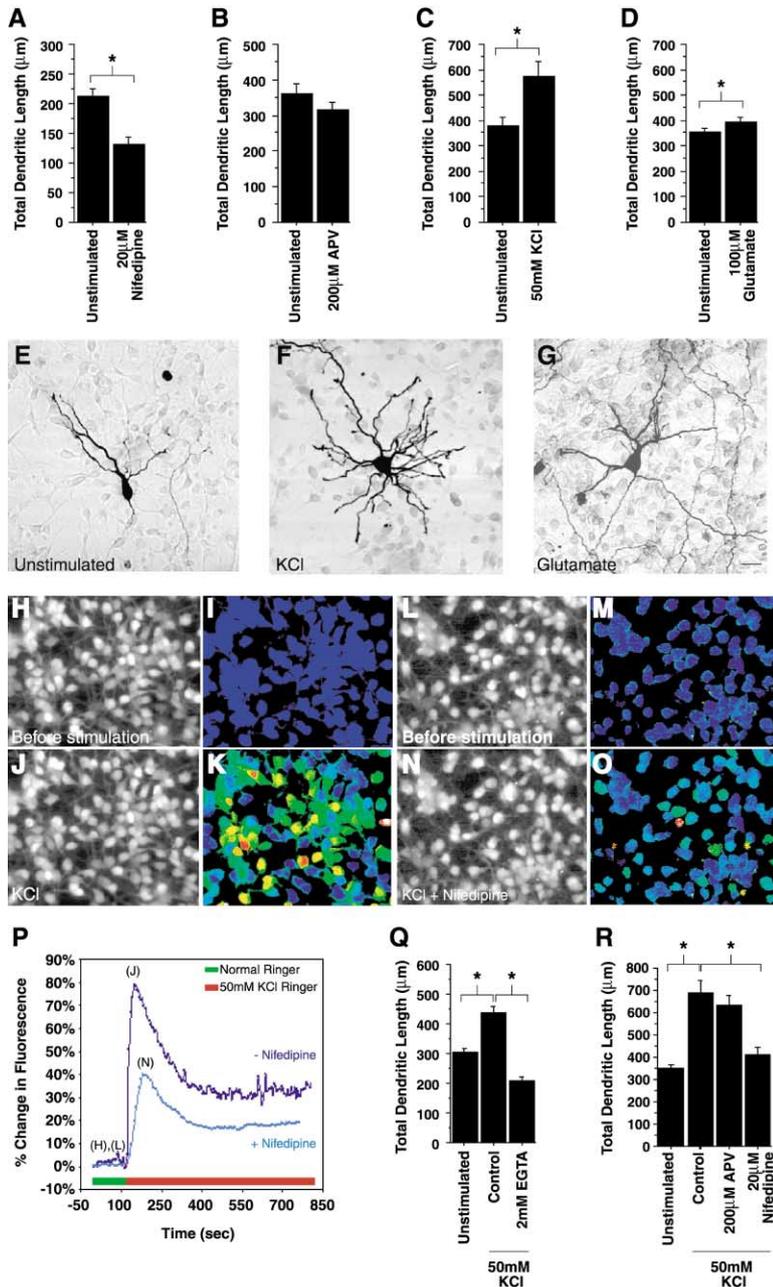
Figure 1. Activation of Voltage-Sensitive Calcium Channels and Calcium Influx Induces Dendritic Growth

(A and B) Effects of blocking VSCCs (with Nifedipine) or NMDA receptors (with APV) on total dendritic length in cortical cultures under basal conditions.

(C and D) Effects of stimulating cortical cultures as indicated on total dendritic length.

(E–G) Examples of E18 cortical neurons in culture at 4 DIV, either unstimulated (E), or stimulated with 50 mM KCl (F) or 100 μM Glutamate (G) as in (C) and (D). Note that neurons depolarized with KCl show markedly increased dendritic growth and branching. Scale bar is 20 μm.

(H–K) Cortical neurons, when depolarized with 50 mM KCl, show an increase in intracellular calcium. Raw fluorescence images of Fura fluorescence in normal saline solution (NSS) at 380 nm before (H) and after stimulation with 50 mM KCl (J). (I) and (K) show colorimetric representations of the data in (H) and (J), respectively, as percentage change. (Blue = 0% change, green = 75% change, yellow = 150% change, red = 250% change.) Note that all four panels show the same group of cells.

(L–O) Nifedipine reduces the intracellular Ca^{2+} increase induced by KCl. Raw fluorescence images of Fura fluorescence in NSS + 20 μM Nifedipine before stimulation (L) and after stimulation with 50 mM KCl (N). (M) and (O) show colorimetric representations of the data in (L) and (N), respectively, as percentage changes. Note that all four panels show the same group of cells.

(P) The percent change in Fura-2 fluorescence ($\Delta F380/F380_{initial}$) is shown as a function of time for cells (n = 14) depolarized with KCl (H–K, dark blue trace) or cells (n = 19) depolarized with KCl in the presence of Nifedipine (L–O).

(Q) Quantification of the effects of chelating extracellular calcium with 2 mM EGTA on KCl-stimulated dendritic growth.

(R) Effect of blocking NMDA receptor (with APV) or voltage-sensitive calcium channels (with Nifedipine) on total dendritic length in unstimulated or KCl-stimulated cortical cultures. Note that KCl-induced dendritic growth, as is intracellular calcium increase, is blocked by Nifedipine, indicating that both are mediated by VSCC activation.

Cortical cultures were transfected at E18 + 2 DIV with either RSV-β-galactosidase or eGFP expression vector and either treated with 20 μM Nifedipine, 200 μM APV, or 100 μM glutamate at 2 DIV for 48 hr or with 50 mM KCl at 3 DIV for 18–24 hr. In experiments combining KCl stimulation and EGTA, APV, or Nifedipine treatment, all agents were added at 3 DIV for 16–24 hr. For calcium imaging experiments, cortical neurons were loaded with Fura-2AM, imaged at E18 + 2 DIV in NSS (see Experimental Procedures) containing 50 mM KCl with or without 20 μM Nifedipine.

To determine if calcium influx was sufficient to drive dendritic growth, we stimulated cortical cultures with 50 mM KCl or 100 μM glutamate, which we have previously shown is effective in activating VSCCs and NMDA receptors, respectively (Hu et al., 1999). Stimulation with KCl, but not with glutamate, led to a dramatic increase in dendritic growth and branching (Figures 1C–1G). To demonstrate that cortical cultures respond to KCl stimulation by increasing intracellular calcium concentration, cultures were depolarized with KCl and calcium dynamics imaged with Fura2 (Figures 1H–1P). In response to KCl stimulation, intracellular calcium rapidly increased and then decayed to approximately 50% of the maximal response (Figure 1P). Chelation of extracellular calcium with EGTA blocked the increase in intracellular calcium (data not shown), indicating that the response to KCl stimulation was dependent on calcium influx. KCl-induced calcium influx was attenuated by Nifedipine, indicating that between 30% and 50% of the depolarization-induced calcium influx is mediated by L-VSCCs (Figures 1L–1P). To determine if the effects of KCl on dendritic growth were mediated by calcium signaling, we examined the consequence of various pharmacological perturbations on KCl-induced den-
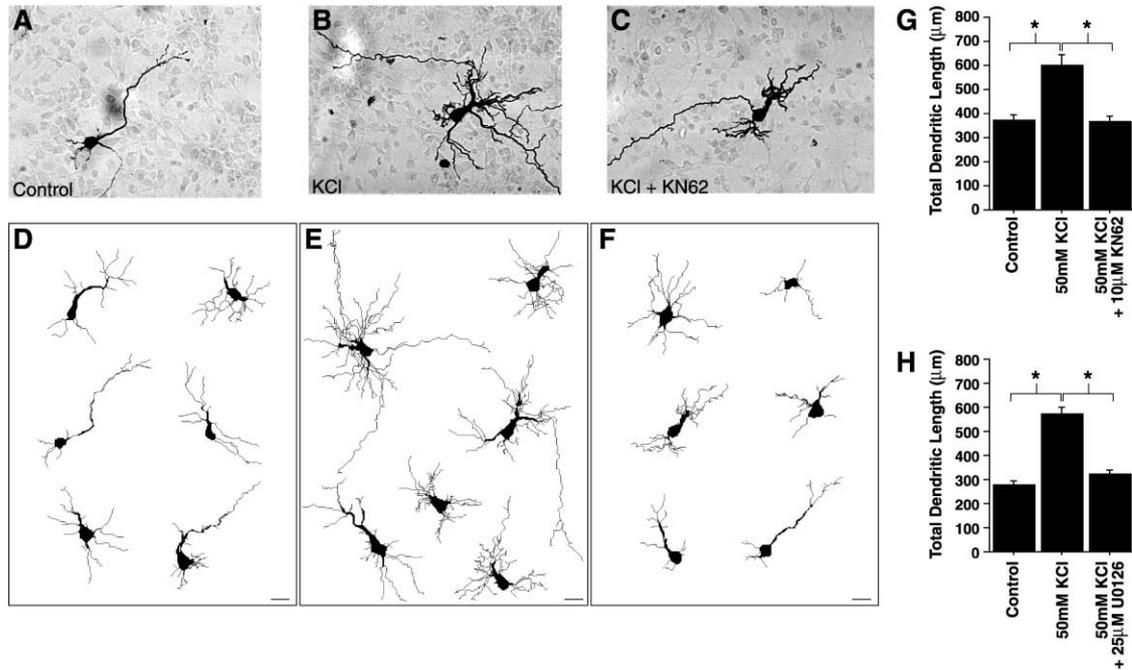
Figure 2. Calcium-Induced Dendritic Growth Is Suppressed by Inhibition of CaM Kinases and MAP Kinases

(A–F) Photomicrographs (A–C) and video-camera lucida drawings (D–F) of E18 cortical neurons left unstimulated, or stimulated with KCl at 3 DIV in the absence or presence of the CaM kinase inhibitor, KN62 (10 μM). Scale bar is 20 μm.

(G and H) Quantification of the effects of inhibiting CaM kinases with KN62 (G) or MAP kinases with U0126 (H) on KCl-induced dendritic growth. Note that inhibiting either pathway attenuates the KCl-induced dendritic growth.

Cortical cultures were transfected at E18 + 2 DIV with RSV-β- galactosidase expression vector, treated as indicated at 3 DIV, and fixed and processed for immunocytochemistry at 4 DIV.

dritic growth. As shown in Figure 1Q, KCl-induced dendritic growth was blocked by EGTA, indicating that the dendritic growth effect is calcium mediated. In addition, KCl-induced dendritic growth was suppressed by Nifedipine, but not APV (Figure 1R). Thus calcium influx via L-VSCCs can activate a signaling pathway that induces dendritic growth.

The principal signaling molecules activated by calcium influx in neurons are the calcium/calmodulin-dependent protein kinases (CaM kinase II and CaM kinase IV), mitogen-activated protein kinases (MAPK), and protein kinase A (PKA) (Ghosh and Greenberg, 1995). Under basal activity conditions, inhibiting either CaM kinases or MAPK, but not PKA, led to an attenuation of dendritic growth, comparable to that seen with VSCC inhibition (see Supplemental Data at http://www. neuron.org/cgi/content/full/34/6/999/DC1). To determine if these signaling molecules were involved in mediating calcium-induced dendritic growth, cortical cultures were stimulated with KCl in the absence or presence of inhibitors of CaM kinases (KN62) or MAPK (U0126). The KCl-induced increase in dendritic growth was suppressed by both KN62 and U0126, indicating that CaM kinase and MAPK contribute to calcium-dependent dendritic growth (Figure 2 and data not shown).

To directly explore the role of CaM kinase II and CaM kinase IV in activity-dependent dendritic development, we expressed wild-type and constitutively active forms of these molecules in cortical neurons. Whereas wild-type forms of CaM kinase II and IV had little effect on dendritic growth, constitutively active mutants that lack the autoregulatory domain had striking but opposite effects on dendritic growth. Expression of a constitutively active form of CaM kinase IV (CaMKIV ca) induced a dramatic increase in dendritic growth, indicating that activation of CaM kinase IV signaling is sufficient to induce dendritic growth (Figures 3A, 3B, and 3D). This increase in dendritic growth occurred in the absence of extracellular stimuli and was similar in magnitude to that seen with KCl stimulation. In contrast, expression of a truncated constitutively active form of CaM kinase II (CaMK IIca) led to a marked reduction in dendritic growth (Figures 3A, 3C, and 3D). We also examined the effects of expressing another constitutively active CaMK II mutant (CaMK II T286D) on dendritic growth. As shown in Figure 3E, expression of this construct also led to a decrease in dendritic growth, but the effects were not as pronounced as those of the truncated CaM kinase II mutant. The difference in the magnitude of the effects of the two CaM kinase II mutants may be due to the fact that the truncated form is targeted to the nucleus, while the point mutant remains in the cytoplasm (Sun et al., 1994). Therefore, although CaM kinase II and CaM kinase IV are both activated by calcium influx, they have distinct effects on dendritic growth. Whereas activation of CaM kinase II restricts or inhibits dendritic growth, CaM kinase IV activation markedly stimulates dendritic growth (Wu and Cline, 1998).

We also examined the effects of transfecting wild-type and constitutively active forms of MEK (a MAP kinase activator) on dendritic development in cortical
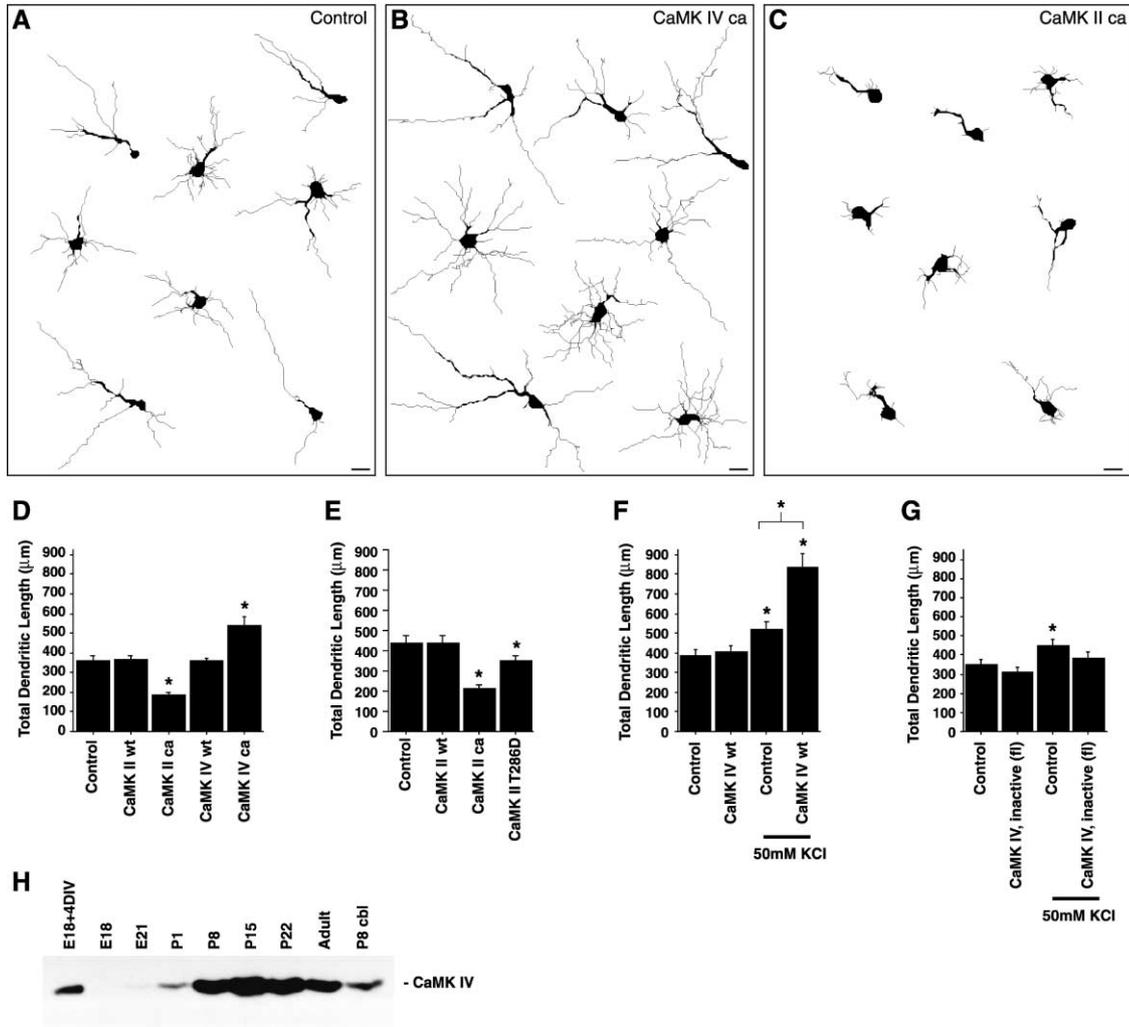
Figure 3. CaM Kinase II and IV Have Distinct Effects on Dendritic Growth

(A–C) Camera lucida drawings of cortical neurons transfected with the parent vector or with constitutively active forms of CaM kinase IV (CaM KIV ca) or CaM kinase II (CaM KII ca). Scale bar is 20 μm.

(D and E) Quantification of the effects of expressing wild-type (wt) or constitutively active (ca) mutants of CaM kinase II and CaM kinase IV on total dendritic length in E18 cortical cultures.

(F) Quantification of total dendritic length in cortical neurons transfected with control vector or wild-type CaM kinase IV in the absence or presence of KCl stimulation. Note that overexpression of CaM kinase IV dramatically potentiates the effect of KCl depolarization on dendritic growth, indicating that intracellular CaM kinase IV levels are limiting for calcium-activated dendritic growth.

(G) Quantification of the effects of expressing a kinase inactive (dominant negative) mutant of CaM kinase IV on KCl-induced dendritic growth.

(H) Expression of CaM kinase IV in the developing rat cerebral cortex as determined by Western blot analysis. Samples in lanes 2–8 are cerebral cortex homogenates from the indicated ages. Lane 1 contains lysates from cortical cultures at E18 + 4 DIV. Lane 9 was loaded with P8 cerebellar homogenate. Note that there is marked increase in CaM kinase IV expression between E18 and P15, which coincides with the period of maximal dendritic growth.

Cortical cultures were transfected at E18 + 2 DIV with RSV-β-galactosidase together with the empty parent vector (control) or the CaM kinase expression plasmids in a molar ratio of 1:2 (βgal:construct), and fixed and processed for immunocytochemistry at E18 + 4 DIV.

neurons. Although pharmacological inhibition of MAPK indicated a role for MAPK in regulating dendritic growth (Figure 2H), expression of constitutively active MEK was not sufficient to induce dendritic growth (data not shown). Since CaM kinase IV was the only kinase sufficient to induce dendritic growth, in the remainder of the study, we focused on the mechanisms that mediate the growth-promoting effects of CaM kinase IV.

To determine if CaM kinase IV expression in the brain is regulated during development, we carried out Western blot analysis of CaM kinase IV expression in cortical

extracts prepared from different developmental ages. As shown in Figure 3H, there was a dramatic increase in CaM kinase IV expression in the cortex between E18 and P15. Remarkably, this almost precisely matches the period of maximum dendritic growth in the cortex (Miller and Peters, 1981), suggesting that CaM kinase IV levels may be causally linked to developmentally regulated dendritic growth. To directly test if intracellular CaM kinase IV levels are limiting for activity-dependent dendritic growth, we examined the consequences of overexpressing wild-type CaM kinase IV on KCl-induced
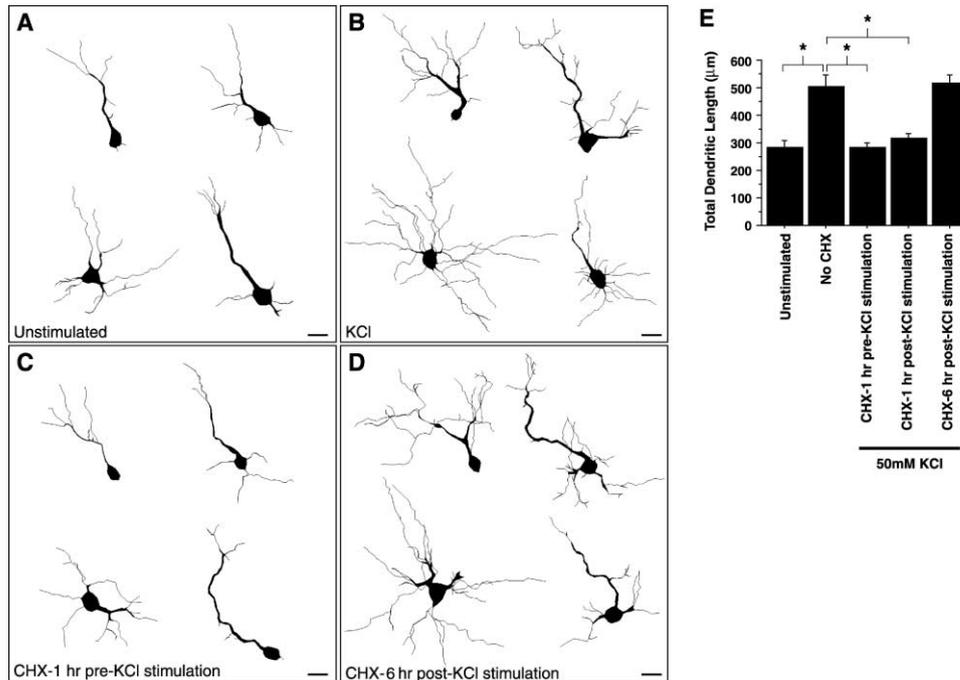
Figure 4. Calcium-Induced Dendritic Growth Requires New Protein Synthesis during a Brief Period Immediately Following Stimulation

(A–D) Camera lucida drawings of cortical neurons transfected with β-galactosidase at E18 + 2 DIV and either left unstimulated (A), stimulated with 50 mM KCl (B), treated with cycloheximide (CHX) 1 hr prior to 50 mM KCl stimulation (C, CHX-1 hr pre-KCl stimulation), or treated with CHX 6 hr after 50 mM KCl stimulation (D, CHX-6 hr post-KCl stimulation). Cultures were fixed and processed for β-galactosidase immunocytochemistry at E18 + 4 DIV. Scale bar is 20 μm.

(E) Quantification of the effects of CHX treatment on KCl-stimulated dendritic growth. Note that CHX blocks KCl-induced dendritic growth when added up to 1 hr after stimulation, but does not inhibit KCl-stimulated dendritic growth when applied 6 hr after stimulation.

dendritic growth. While overexpression of wild-type CaM kinase IV did not significantly affect dendritic growth in unstimulated neurons, it greatly potentiated the calcium-dependent increase in dendritic growth in KCl-stimulated neurons (Figure 3F). This result indicates that the level of expression of CaM kinase IV is a key determinant of calcium-dependent dendritic growth.

As a final test of the role of CaM kinase IV in dendritic development, we examined the effects of a kinase-dead (putative dominant negative) form of CaM kinase IV (Ho et al., 1996) on calcium-dependent dendritic growth. As shown in Figure 3G, the effect of KCl stimulation on dendritic growth was attenuated by expression of this construct, supporting a role for CaM kinase IV in activity-dependent dendritic growth. The attenuation of activity-dependent dendritic growth by KN62, together with the effects of altering CaM kinase IV levels and activity on dendritic growth, strongly suggests that CaM kinase IV is a major effector of calcium-dependent dendritic growth in cortical neurons.

Calcium signals could influence dendritic morphology either by inducing local cytoskeletal changes or by regulating the synthesis of proteins that control dendritic growth. Since CaM kinase IV is best characterized for its role in transcriptional regulation, the involvement of CaM kinase IV suggests that calcium-dependent dendritic growth may require new protein synthesis. To test this possibility directly, we treated cortical cultures with the protein synthesis inhibitor cycloheximide, either immediately prior to stimulation or at various times after

KCl stimulation. As shown in Figures 4A–4C and 4E, KCl-induced dendritic growth was completely prevented by pretreatment with cycloheximide, indicating that calcium-dependent dendritic growth requires new protein synthesis. When cycloheximide was added 1 hr after stimulation, dendritic growth was also inhibited, but addition of cycloheximide 6 hr after stimulation had no inhibitory effect on dendritic growth (Figures 4D and 4E). Thus, activity-dependent dendritic growth requires new protein synthesis between 1 and 6 hr following stimulation, suggesting that calcium-induced dendritic growth may have a transcriptional component.

The best-characterized transcription factor target of CaM kinase IV is cAMP response element binding protein (CREB) (Sun et al., 1994; Matthews, 1994; Sun et al., 1996). CaM kinase IV induces CREB-mediated transcription by phosphorylation of CREB at Ser133, which is required for CREB to associate with its coactivator, CBP (Hu et al., 1999; Chawla et al., 1998). We therefore carried out a series of experiments to examine the role of CREB and CBP in calcium and CaM kinase IV-induced dendritic growth. To determine if CREB function was required for activity-dependent dendritic growth, we examined the consequences of expressing a dominant negative mutant of CREB in cortical neurons (Walton et al., 1992). As shown in Figure 5A, KCl-induced dendritic growth was completely blocked by dominant negative CREB, indicating that calcium-dependent dendritic growth requires CREB function. Since we propose that CaM kinase IV is a key mediator of activity-dependent
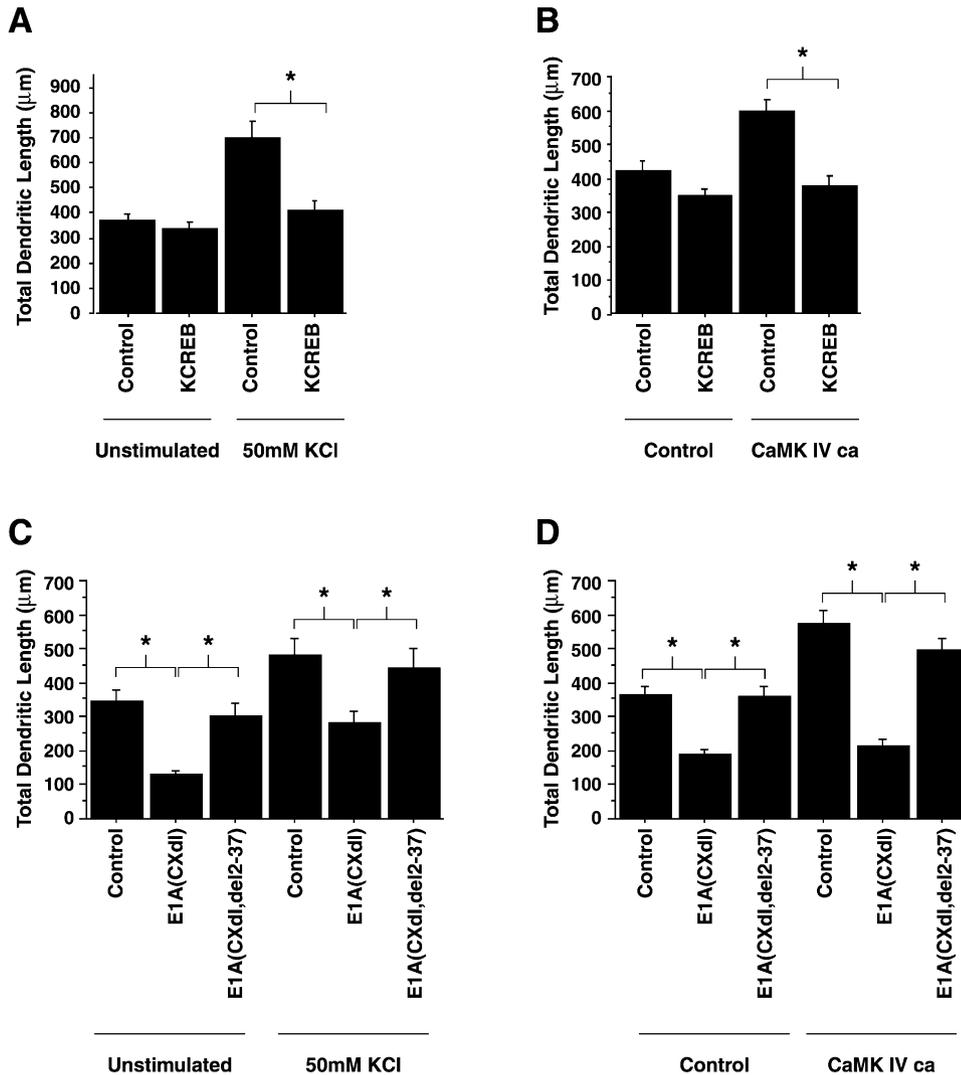
A

B

C

D

Figure 5. Calcium Influx and CaM Kinase IV Activation Induce Dendritic Growth via CREB- and CBP-Mediated Transcription

(A) Quantification of the effects of expressing a dominant negative mutant of CREB (KCREB) on KCl-induced dendritic growth.

(B) Quantification of the effects of expressing a KCREB on dendritic growth induced by constitutively active CaM kinase IV.

(C) Quantification of the effects of expressing a dominant inhibitor of CBP (E1A CXdl) or a mutant of the inhibitor that does not bind CBP (E1A CXdl, del 2-37) on KCl-induced dendritic growth.

(D) Quantification of the effects of expressing E1A (CXdl) and E1A(CXdl, del 2-37) on dendritic growth induced by constitutively active CaM kinase IV.

Cortical cultures were transfected at E18 + 2 DIV with RSV-β-galactosidase, CaM kinase IV ca, and either empty parent vector, KCREB, E1A(CXdl), or E1A(CXdl, del 2-37) at molar ratios of 1:2:8 (β-gal:CaM kinase IV ca:construct) and at E18 + 4 DIV were fixed and processed for immunocytochemistry.

dendritic growth, we also examined the effect of dominant negative CREB on CaM kinase IV-induced dendritic growth. This too was completely blocked by dominant negative CREB (Figure 5B). These experiments show that calcium and CaM kinase IV induce dendritic growth via CREB-mediated transcription.

We and others have previously shown that calcium activation of CREB-dependent transcription requires CBP function, and that CaM kinase IV phosphorylates CBP and induces CBP-mediated transcription (Chawla et al., 1998; Hu et al., 1999). We therefore tested the role of CBP in calcium and CaM kinase IV-induced dendritic growth by expressing a mutant form of the E1A gene

(E1A (CXdl)) that inhibits CBP function in cortical neurons (Hu et al., 1999). As shown in Figures 5C and 5D, the ability of KCl and CaM kinase IV to induce dendritic growth was completely blocked by E1A (CXdl). The fact that the effects of inhibiting CBP were more pronounced than the effects of inhibiting CREB may be due to the fact that CBP is known to interact with many different transcription factors, and therefore inhibiting CBP might inhibit transactivation via transcription factors in addition to CREB. To determine if the E1A effect was indeed due to its ability to bind CBP, we tested the effect of a slightly variant mutant (E1A (CXdl del 2-37)) that can no longer bind to CBP (Sun et al., 1994). This E1A construct
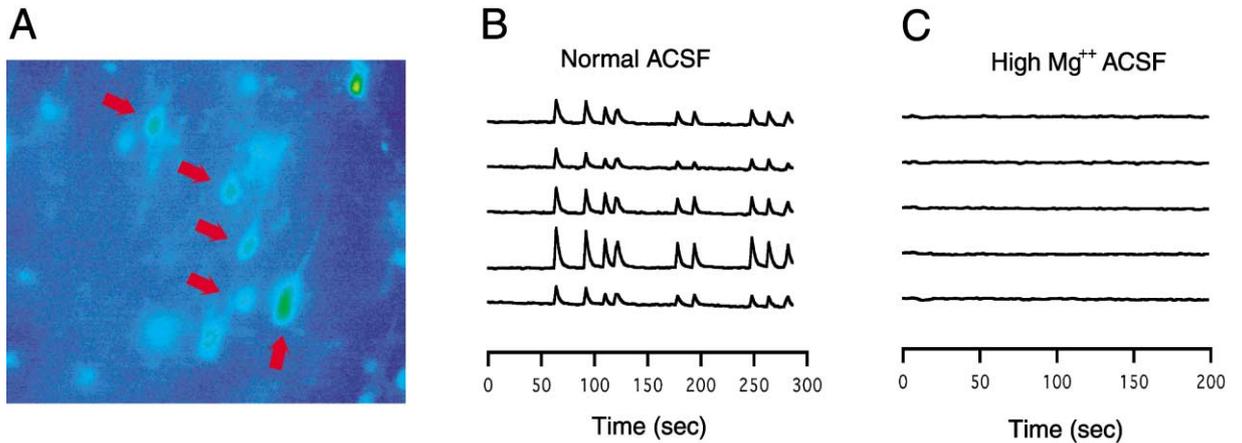
Figure 6. Neurons in Cortical Slice Cultures Show Spontaneous Calcium Transients

(A) Example of cortical neurons in slice culture loaded with Fura2-AM. Note that the cell bodies of individual Fura-loaded neurons can be clearly distinguished.

(B) Examples of spontaneous somatic calcium transients in cortical neurons in slice culture. The change in Fura-2 fluorescence (ΔF380/F380$_{initial}$) is shown as a function of time. Note that calcium transients in groups of neurons are synchronized, as reported by Mao et al. (2001). These recordings are in ACSF with 3 mM Ca/0 mM Mg.

(C) Spontaneous transients are eliminated in ACSF containing 1 mMCa/3mM Mg, as well as by the calcium chelator EGTA (data not shown).

was no longer effective in blocking KCl- and CaM kinase IV-induced dendritic growth. Therefore, as would be expected for a CREB-dependent mechanism, activity-dependent dendritic growth requires CBP function.

In a final series of experiments, we investigated whether the signaling pathway we had identified based on KCl-induced dendritic growth in cortical cultures was likely to be involved in dendritic growth control in a physiological context. For these experiments we turned to postnatal cortical slice cultures, which we and others have shown preserve cortical cytoarchitecture (Whitford et al., 2002). To determine if neurons in such slice cultures were spontaneously active, we imaged calcium transients in postnatal cortical slices using Fura-2 imaging. As shown in Figure 6, individual neurons can clearly be visualized based on Fura-2 loading, and many of them show calcium transients. As reported by Yuste and colleagues, groups of cortical neurons are phase-locked in their calcium responses (Figure 6B). These transients are eliminated in a high Mg^{2+}/low Ca^{2+} bath (Figure 6C), or by treatment with EGTA (data not shown), consistent with the interpretation that the transients indeed reflect calcium signals. It has previously been shown that these transients correspond to action potentials-induced calcium influx (Mao et al., 2001). Thus, cortical neurons in slice cultures have a fairly high degree of spontaneous activity, which makes them suitable for studies of activity-dependent dendritic growth.

The two principal conclusions of our cell culture experiments were that calcium influx via L-VSCCs induces dendritic growth, and that this requires CREB-mediated transcription. To determine if these key features were also true for dendritic growth in cortical slices, we first examined whether L-VSCC activation regulated CREB phosphorylation in cortical slices under spontaneous activity conditions. Western blots indicated that phosphorylated CREB could be readily detected in untreated

cortical slices (Figure 7C). Treatment of the cultures with the L-VSCC blocker Nifedipine significantly reduced CREB phosphorylation (Figure 7C), indicating that endogenous activation of L-VSCCs regulates phosphorylation of CREB in cortical slice cultures.

To determine if signaling via L-VSCCs regulates dendritic growth in cortical slice cultures, we transfected P7 cortical slice cultures with a GFP expression vector. The slices were then placed in control media, or in media containing the L-VSCC inhibitor Nifedipine for 2 days. At the end of the experiment, slices were fixed and processed for GFP immunofluorescence, which reveals the morphologies of individual transfected neurons (Whitford et al., 2002). For this set of experiments, we restricted our analysis to layer 5 cells, which are a population of cells that have well-developed dendrites at this age. As shown in Figure 7, cortical neurons in slice cultures have well-developed basal dendritic trees that resemble their morphology in vivo. Treatment with Nifedipine led to a significant reduction in total dendritic length, indicating that calcium signaling via spontaneous L-VSCC activation in cortical slice contributes to dendritic growth. Sholl analysis of the dendritic trees in this experiment also revealed a significant reduction in the number of crossings in the presence of Nifedipine (Figure 7E), indicating that calcium signaling increases the overall complexity of the dendritic tree rather than simply extending the length of existing dendrites.

Finally, we examined the consequence of inhibiting CREB function on dendritic development in cortical slice cultures. For these experiments, P7 cortical slices were transfected with GFP together with a control vector or a dominant negative CREB construct (KCREB). As shown in Figures 7F–7I, total dendritic length and dendritic complexity were sharply reduced in cells expressing dominant negative CREB. Thus, as surmised from the cell culture experiments, CREB-mediated transcription plays an important role in regulating cortical den-
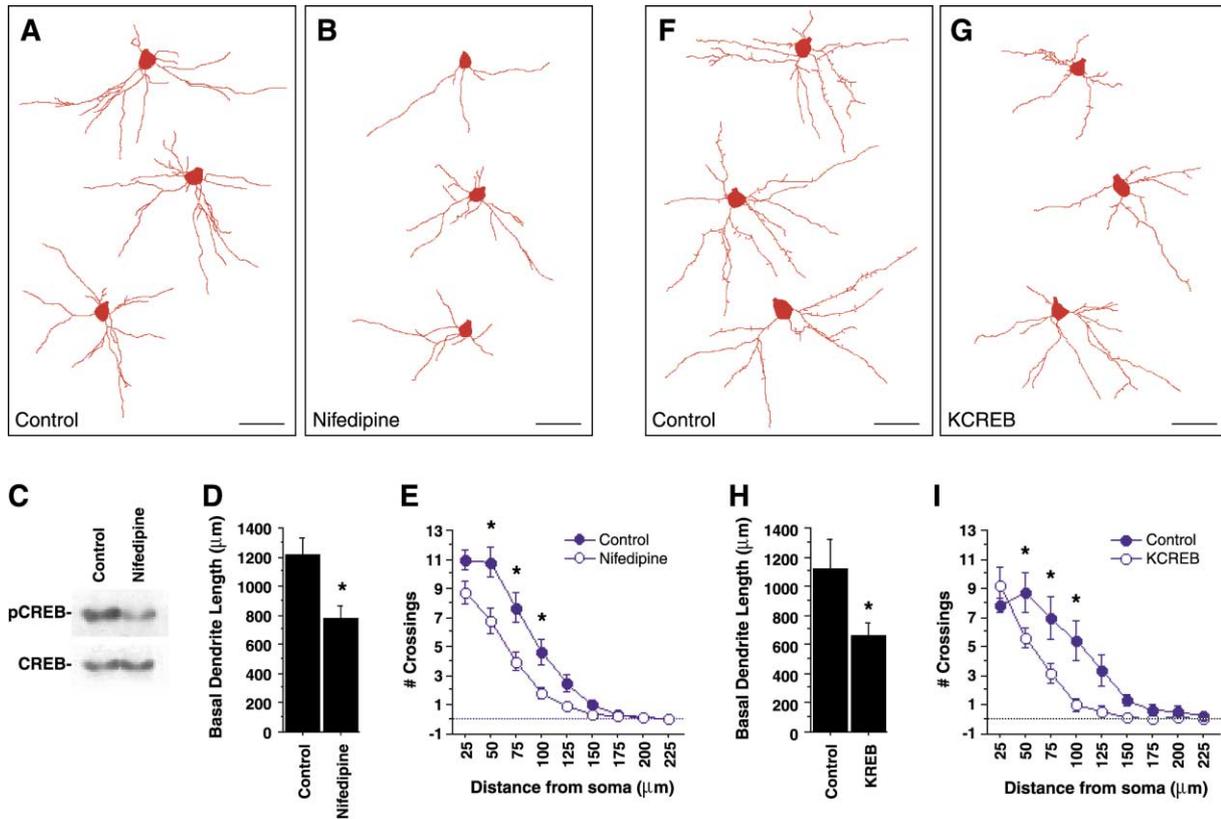
Figure 7. Inhibition of VSCCs and CREB Activity Attenuates the Growth and Complexity of Layer 5 Pyramidal Neuron Basal Dendrites in Cortical Slice Cultures

(A and B) Camera lucida drawings of basal dendrites of GFP-transfected layer 5 pyramidal neurons in P7 cortical slices at 2 DIV that were either unstimulated (A) or treated with 20 μM Nifedipine (B) for 36 hr. Scale bar is 50 μm.

(C) Level of CREB phosphorylation as detected by phospho-CREB Western blot in P7 cortical slice cultures under basal conditions (Control) or after treating with 20 μM Nifedipine (Nifedipine) for 36 hr. Note that inhibiting L-VSCCs reduces phospho-CREB levels in cortical slice cultures.

(D and E) Effect of Nifedipine treatment on the complexity of layer 5 basal dendrites as measured by total dendrite length (D) and Sholl analysis (E). Asterisks indicate $p < 0.05$.

(F and G) Camera lucida drawings of basal dendrites of GFP-transfected layer 5 pyramidal neurons in P7 cortical slices at 3 DIV that were transfected with GFP together with either empty parent vector (F) or a dominant negative mutant of CREB (KCREB) (G) for 48 hr. Scale bar is 50 μm.

(H and I) Effects of expressing parent vector or KCREB on the complexity of layer 5 basal dendrites as measured by total dendrite length (H) and Sholl analysis (I). Note that expression of KCREB decreased both the length and complexity of basal dendrites.

drite development. Together with the Nifedipine experiments, these observations support a model in which calcium influx through L-VSCCs regulates dendrite development via CREB-mediated transcription.

Discussion

Our observations suggest that calcium influx induces dendritic growth via activation of a transcriptional program that involves activation of CaM kinase IV, and CREB- and CBP-mediated gene expression. MAP kinase is also likely to participate in this process since inhibitors of MAP kinase block calcium-induced increases in dendritic growth. MAP kinase has previously been implicated in activity-dependent filopodia formation in hippocampal neurons (Wu et al., 2001). Thus, MAP kinase signaling appears to be involved both in mediating the short-term effects of calcium signaling,

such as filopodia formation, as well as longer term effects such as regulation of dendritic growth.

While both CaM kinase IV and MAP kinase appear to be required for calcium-induced dendritic growth, it is intriguing that a constitutively active form of CaM kinase IV, but not of MEK, can induce dendritic growth. Although the basis of the difference in the effects of CaM kinase IV and MAP kinase is not yet known, one possibility is that it may be related to their ability to induce CREB/CBP-mediated transcription. Both CaM kinase IV and MAP kinase have been implicated in mediating calcium activation of CREB-dependent transcription, but they are thought to differ in their mechanism of action. While CaM kinase IV appears to be involved in the rapid phosphorylation of CREB, the MAP kinase pathway is activated with slower kinetics and appears to be important for the sustained phosphorylation of CREB (Shaywitz and Greenberg, 1999; Dolmetsch et al., 2001; Hardingham et al., 2001; Chawla and Bading,

2001). Both the rapid and sustained components of CREB phosphorylation are required for transcription (Dolmetsch et al., 2001). In addition, calcium signaling directly activates CBP, which is another important component of the CREB transcription complex (Chawla et al., 1998; Hu et al., 1999; Impey et al., 2002; but see Kornhauser et al., 2002). In our experiments, the fact that inhibitors of either CaM kinases or MAP kinase block calcium-dependent dendritic growth is consistent with the dual requirement of these kinases in activation of CREB. Constitutively active CaM kinase IV is probably effective in inducing dendritic growth because it can sustain CREB phosphorylation due to its continued presence in transfected neurons, and it is effective in activating CBP (Hu et al., 1999; L.R. and A.G., unpublished observations). Activation of MEK alone, in contrast, may not be effective in inducing dendritic growth because of its inability to activate CBP (Hu et al., 1999). Thus, the differential ability of CaM kinase IV and MEK to activate CBP may be the molecular distinction that underlies the distinct biological effects of these kinases.

The target genes of CaM kinase IV and CREB that mediate dendritic growth are not known, but an important potential target is brain-derived neurotrophic factor (BDNF). We have previously shown that CaM kinase IV can induce BDNF expression by a CREB-dependent mechanism (Shieh et al., 1998), and it has been reported that BDNF expression in cortical neurons can lead to increased dendritic growth (McAllister et al., 1995, 1997; Horsch et al., 1999). Thus calcium- and CaM kinase IV-induced dendritic growth may be mediated by BDNF. To test this possibility, it will be important to determine if calcium-induced dendritic growth is compromised in mice lacking BDNF or the BDNF receptor, Trk-B. Regulation of dendritic growth via CaM kinase IV, CREB, and BDNF may be relevant to mechanisms of learning and memory since all of these proteins have been previously implicated in mediating different aspects of long-term plasticity (Dash et al., 1990; Bito et al., 1996; Shaywitz and Greenberg, 1999). Since dendritic architecture is likely to influence patterns of synaptic connectivity, some of the effects of calcium signaling on long-term plasticity may be mediated by the CaM kinase IV- and CREB-dependent regulation of dendritic growth.

While CREB is the best-characterized target of CaM kinase IV, it is likely that activation of CREB alone is not sufficient to induce dendritic growth. In preliminary studies, we have found that, in contrast to the effects of constitutively active CaM kinase IV, expression of a constitutively active form of CREB is not sufficient to induce dendritic growth by itself (L.R. and A.G., unpublished observation). Thus the effects of CaM kinase IV on dendritic morphology are likely to involve activation of additional transcription factors. The identification of these putative CaM kinase IV targets should provide important insight into the mechanisms that underlie the cellular effects of CaM kinase IV.

A major goal of future experiments will be to assess the role of L-VSCCs, CaM kinase IV, and CREB in regulation of dendritic growth in vivo. Along with developing appropriate pharmacological or genetic methods to perturb these molecules, this will require a better understanding of how and when calcium signals influence dendritic growth during development. While it is clear that dendritic growth is regulated by a combination of influences that include intrinsic genetic programs, extracellular growth factors, and neural activity, their relative contributions in regulating specific aspects of dendritic patterning are not well understood. Based on the expression patterns of various extracellular factors and the timing of innervation, it seems likely that intrinsic genetic programs and extracellular factors are most important during embryonic development, and that activity becomes progressively more important during postnatal development. While this is a reasonable speculation, it has not been rigorously tested. Selective inactivation of L-VSCC, CaM kinase IV, or CREB during specific periods of development should help address these issues.

While we have focused on the role of transcription-dependent mechanisms in this study, it is likely that local calcium signaling events also play an important role in dendritic patterning. In addition to previous studies that support a role of NMDA receptor signaling in regulation of spine morphology (Maletic-Savatic et al., 1999; Engert and Bonhoeffer, 1999), a recent study suggests that local calcium signaling can play an important role in regulating the stability of terminal dendritic segments. Lohmann et al. (2002) report that in retinal ganglion cells, distal dendritic segments display localized calcium transients due to calcium release from intracellular stores. Suppression of these calcium signals leads to the rapid withdrawal of distal dendritic segments. Therefore, the effects of activity on dendritic morphology are likely to involve both local and transcriptional consequences of calcium signaling. The fact that cycloheximide suppresses calcium-dependent dendritic growth in our experiments is consistent not only with a role for calcium-induced gene expression, but also with a potential role for local protein synthesis in this process. It will be interesting to determine whether local protein synthesis contributes to the cellular effects of local dendritic calcium transients.

The observation that calcium influx may influence dendritic development by both transcription-dependent and transcription-independent processes raises the possibility that calcium influx via NMDA receptors and VSCCs may regulate separate aspects of activity-dependent dendritic growth and patterning. While the overall growth of the dendritic arbor may be regulated by L-VSCC activation and CREB-dependent transcription, the stability of particular dendritic branches may depend upon NMDA receptor-dependent activation of intracellular stores . A related possibility is that NMDA receptor activation may lead to local biochemical changes that in effect tag regions of the dendrite for subsequent growth. L-VSCC-dependent events may then specifically drive dendritic growth at these tagged sites. Such a mechanism would allow for targeted dendritic growth in a transcription-dependent manner. This possibility is conceptually similar to the synaptic tag hypothesis that has been proposed for long-term changes in synaptic strength (Frey and Morris, 1997).

The recent identification of a number of signals that influence dendritic development suggest a model in which different signals regulate particular aspects of dendritic growth and patterning. Studies on the development of cortical neurons serve as a useful illustration of this point. Soon after arriving in the cortical plate, neu-

rons are exposed to a gradient of Sema 3A (Polleux et al., 2000). This signal acts as a chemorepellant for efferent axons and as a chemoattractant for apical dendrites, thus establishing the core morphology of cortical pyramidal neurons. The subsequent growth of cortical dendrites is regulated by a number of extracellular signals. Important among these are BDNF and Slit-1, which act as growth and branching factors, and Notch, which appears to restrict growth (McAllister et al., 1995, 1997; Sestan et al., 1999; Redmond et al., 2000; Whitford et al., 2002). In addition to these extracellular factors, activity-dependent calcium influx is likely to exert a considerable influence on dendritic morphology. We propose that activation of L-VSCCs regulates overall dendritic growth via a CaM kinase IV and CREB-dependent mechanism, and, by analogy with retinal ganglion cells, the stability of individual dendritic branches may be regulated by local elevations in intracellular calcium. Thus the coordinated action of both activity-independent and activity-dependent mechanisms may specify the mature morphology of cortical neurons.

Experimental Procedures

Dissociated Cell Cultures and Transfections
Cortical neurons from Long Evans rats were cultured as described previously (Threadgill et al., 1997). Briefly, embryonic day 18 cortical cells were digested in 10 U/ml papain (Worthington), dissociated, plated onto poly-D-lysine and laminin-coated 12 well dishes at 1×10^6 cells per well in glutamine-free Basal Media Eagle, 1 mM glutamine, 1% N2, and fetal bovine serum (all from Life Technologies), and maintained at 37°C in 95%/5% O_2/CO_2. Cells were transfected by a modified calcium phosphate procedure as described (Threadgill et al., 1997) at 2 days in vitro (DIV) and processed for β-galactosidase immunocytochemistry at 4 DIV with either mouse anti-β-galactosidase (Promega) or rabbit anti-β-galactosidase (5′–3′) primary antibodies and visualized with peroxidase conjugated secondary antibodies (ABC Elite, Vector Labs) (Threadgill et al., 1997). The wild-type and truncated CaM kinase II and IV constructs and the CaM kinase IV inactive expression construct used in this experiment have been previously described (Sun et al., 1994). In all cases, transfection with the empty parent vector was used as control.

Analysis of Dissociated Cell Cultures
Transfection experiments were carried out in duplicate wells, and all of the experiments described were repeated multiple times with comparable results. Images of RSV-β-galactosidase-transfected neurons were captured using a digital CCD camera (Dage, MTI) attached to the side port of an inverted microscope driven by IP Lab Spectrum 3.1.1 image-acquisition software (Scanalytics). Captured cells were then traced using ClarisDraw software. To assess the effects of pharmacological treatments and expression constructs on dendritic growth, the dendritic trees of at least 25 neurons were reconstructed and the total dendritic length was determined using IP Lab Spectrum software. Data are shown as mean ± standard error. Asterisks indicate statistically significant differences (p < 0.05).

Calcium Imaging in Dissociated Cell Cultures
Dissociated cortical neurons were cultured on poly-lysine/laminin coated 35 mm glass bottom tissue culture dishes (MatTek) at 1.5×10^6 cells per dish and imaged after 2 DIV. On the day of imaging, cells were washed twice with DMEM and incubated with 2.5 μM Fura-2AM (Molecular Probes) in DMEM for 15 min at 37°C. The cells were washed twice with DMEM and left at 37°C for another 15 min to allow for complete hydrolysis of the acetoxymethyl (AM) ester moiety. In experiments where Nifedipine was added, cells were incubated with 20 μM Nifedipine (Calbiochem) during this second DMEM incubation. In these experiments, all subsequent solutions

used contained 20 μM Nifedipine. In all experiments, the cells were imaged in Normal saline solution (NSS: 137 mM NaCl, 25 mM Glucose, 10 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$). In experiments where cells were depolarized, osmotic stress was prevented by isosmotically substituting 50 mM KCl for 50 mM NaCl. Solutions were changed using a gravity-based perfusion system with flow rates approximately 2–3 ml/min. Imaging was always completed within 1 hr of loading. Images of Fura-loaded cells were captured at 0.2–0.5 Hz with an Orca II digital camera (Hamamatsu Photonics) attached to the side port of a Nikon Eclipse TE300 microscope driven by OpenLab 3 Imaging software (Improvision, Inc). Each experiment was repeated a minimum of three times and yielded comparable results.

Slice Cultures and Transfections
Cortices from postnatal day 7 rats were coronally sectioned in ice-cold HBSS on a vibratome (Ted Pella) at 350 μm and transferred onto a porous membrane (Becton-Dickinson). Slices were maintained at an air-media interface in 50% glutamine-free Basal Media Eagle, 25% horse serum, 20 mM glucose, 1 mM glutamine 1 mM Hepes, and HBSS as previously described (Whitford et al., 2002). Individual neurons were transfected with green fluorescent protein (GFP, Clonetech) plasmid-coated 1.6 μm gold particles using biolistics (Helios gene gun, Bio-Rad) on the day of plating and at 1 DIV, were treated for 36 hr with either 20 μM Nifedipine (Calbiochem) or vehicle then fixed in 4% paraformaldehyde. Cotransfections were similarly performed with 1.6 μm gold particles coated with GFP and either KCREB or empty vector at molar ratios of 1:4 (GFP:construct) and fixed after 48 hr. Slices were processed for GFP and MAP2 immunofluorescence with rabbit anti-GFP (Molecular Probes) and mouse anti-MAP2 (Sigma) primary antibodies, visualized with Alexa488 and Alexa568 conjugated secondary antibodies (Molecular Probes), and counterstained with Hoechst. Hoechst and MAP2 immunostaining were used to identify layer 5, from which GFP-transfected pyramidal neurons were chosen for morphological analysis.

Analysis of Slice Cultures
To analyze the morphology of transfected neurons in cortical slice cultures, the entire basal dendritic tree of layer 5 pyramidal neurons was analyzed by z sectioning on a Zeiss LSM510 confocal microscope. Z stacks were imported into OpenLab 3 Imaging software (Improvision, Inc.) and the dendrite length in the x and y planes measured. The measurements do not include corrections for inclinations of dendritic processes and therefore represent projected lengths. Sholl analysis was carried out by counting the number of basal dendrites that cross a series of concentric circles at 25 μm interval distances from the cell soma. The representative basal dendritic trees shown in Figure 7 were traced in OpenLab. Data are shown as mean ± standard error. Asterisks indicate statistically significant differences (Student's t test, p < 0.05).

Calcium Imaging in Cortical Slices
Calcium imaging of spontaneous calcium transients was performed on rat cortical slices (Mao et al., 2001). Briefly, rat pups ranging in age from P7–P14 were quickly decapitated. The whole brain was removed and mounted on a vibratome cutting block in an ice cold bath of ACSF bubbled with 95% O_2 and 5% CO_2. (ACSF composition follows in mM: 123 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 glucose, 6 KCl, 1 $CaCl_2$, 2 $MgCl_2$.) Brain sections were cut approximately 300–350 μm in thickness and cultured for 1 day in serum containing media at 37°C in an incubator. Immediately before imaging, slices were loaded with 10–15 μM Fura-2AM (Molecular Probes) for 1 hr at 37°C and then washed with ACSF for 10–15 min to reduce background fluorescence. The slice was then loaded onto the stage of a Nikon Eclipse TE300 inverted scope and visualized using a 40× oil immersion objective (Nikon). Solutions were changed using a gravity-based perfusion system with flow rates around 3–5 ml/min. Images were acquired using a Hammatsu Orca digital camera driven by Openlab Software (Improvision). Frames were acquired at 1 Hz for 300–500 s. After a solution change, the slices were allowed to equilibrate in new solutions for several minutes before recording began.

Western Blot Analysis

Cortices were dissected from embryonic (E18 and E21) and postnatal rats (P1, P8, P15, P22, and adult) or samples collected from E18 cultured cells. Tissues and cells were homogenized with a dounce homogenizer in lysis buffer as previously described (Redmond et al., 2000). Protein concentration was determined using BCA protein assay (Pierce) and 10 μg of each homogenate was separated by PAGE and transferred to nitrocellulose. Blots were blocked in 5% milk in TBST and incubated with anti-CaMK IV antibody (Transduction Labs) diluted in blocking solution for 16 hr at 4°C. Blots were incubated in peroxidase conjugated anti-mouse secondary antibody (Amersham) diluted in blocking solution and visualized using a chemiluminescent detection system (Pierce). Cortical slice cultures were harvested by boiling SDS lysis, proteins separated on SDS-PAGE, and transferred to nitrocellulose using standard protocols. Blots were blocked in 5% milk in TBST and incubated with anti-phospo-CREB antibodies (Cell Signaling) diluted in blocking solution for 16 hr at 4°C. Secondary antibody incubation and visualization were performed as above. After visualization of phospho-CREB levels, blots were stripped and reprobed with anti-CREB (phosphorylation-state independent, Cell Signaling) antibodies diluted in 4% horse serum in TBST and visualized as above.

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