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Signaling Mechanisms Underlying Reversible, Activity-Dependent Dendrite Formation

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Summary

Neuronal activity and neurotrophins play a central role in the formation, maintenance, and plasticity of dendritic arbors. Here, we show that neuronal activity, mediated by electrical stimulation, KCl depolarization, or cholinergic receptor activation, promotes reversible dendrite formation in sympathetic neurons and that this effect is enhanced by NGF. Activity-dependent dendrite formation is accompanied by increased association of HMW MAP2 with microtubules and increased microtubule stability. Inhibition of either CaMKII or the MEK-ERK pathway, both of which phosphorylate MAP2, inhibits dendrite formation, but inhibition of both pathways simultaneously is required for dendrites to retract. These data indicate that neuronal activity signals via CamKII and the ERKs to regulate MAP2:microtubule interactions and hence reversible dendrite stability, and to provide a mechanism whereby activity and neurotrophins converge intracellularly to dynamically regulate dendritic morphology.

Introduction

The morphological differentiation of dendrites plays a key role not only in the developmental establishment of circuitry, but also in activity-and experience-dependent neural plasticity. Two extrinsic cues known to modify the formation and maintenance of dendrites are neuronal activity (Volkmar and Greenough, 1972; Katz et al., 1989; Bailey and Kandel, 1993) and the neurotrophin family of growth factors (reviewed in McAllister et al., 1999). Interactions between these two cues have been particularly well studied in the developing visual cortex, where neurotrophins have been shown to regulate the morphology of cortical dendrites, at least partially, via a mechanism that requires neuronal activity, and where neurotrophins have been implicated in the activity-dependent segregation of lateral genicule axons into ocular dominance columns (reviewed in McAllister et al., 1999). Moreover, a recent study (Xu et al., 2000) indicates that neurotrophin signaling via the TrkB receptor is essential for the maintenance of dendritic arbors of at least some mature cortical neurons. Thus, neurotrophins and activity collaborate to determine dendritic morphology, and this interaction is apparently essential for the development and plasticity of central neural circuitry.

Sympathetic neurons of the peripheral nervous system provide one accessible model system for studying the cellular mechanisms that allow neurotrophins and neuronal activity to promote dendritogenesis. Prior to developmental target contact, sympathetic neurons make rudimentary dendrites and their incoming afferents, sympathetic preganglionic axons, form synapses on sympathetic cell bodies (Rubin, 1985a, 1985b). After target contact and initial establishment of a circuit, sympathetic dendrites grow extensively, and many of the preganglionic synapses rearrange themselves onto these new dendrites. Interestingly, sympathetic dendrites continue to grow in size throughout the animals life, likely as a response to the increased target territory that any single neuron must innervate as the animal grows (reviewed in Purves et al., 1988; Purves, 1988). Moreover, the dynamic retraction and formation of sympathetic dendrites has been imaged in living adult animals (Purves et al., 1986).

A series of classic in vivo studies established that interactions between sympathetic neurons and their targets were essential for dendrite formation and maintenance in this system. Axotomy and the subsequent disconnection from targets caused retraction of sympathetic dendrites (Purves, 1975; Yawo, 1987), while experimental manipulations that increased target size led to a corresponding increase in the size of the dendritic arbor (Voyvodic, 1989). Evidence that target-derived NGF was a major determinant of these effects came from studies showing that injection of anti-NGF in adult rats caused dendritic retraction (Ruit and Snider, 1991), while systemic treatment of neonatal or adult rats with exogenous NGF caused dramatic expansion of dendritic arbors (Snider, 1988; Ruit et al., 1990). However, although it is clear that NGF and target connectivity are critical for dendrite formation and maintenance in this system, the underlying cellular mechanisms are undefined.

In this paper, we have taken advantage of cultured sympathetic neurons to address this issue. Under basal culture conditions, NGF promotes survival and axonal growth from sympathetic neurons, but is insufficient to promote dendrite formation. Here, we show that neuronal activity promotes reversible dendrite formation in cultured sympathetic neurons and that this effect is enhanced by NGF. The activity-dependent dendrite formation is accompanied by increased association of HMW MAP2 with microtubules and a resultant increase in microtubule stability. Inhibition of either CaMKII or the MEK-ERK pathway, both of which can phosphorylate MAP2, inhibits activity-dependent dendrite formation, and simultaneous blockage of these two pathways will cause preformed dendrites to retract. Thus, neuronal activity and NGF converge on CaMKII and the ERKs to dynamically regulate MAP2:microtubule interactions

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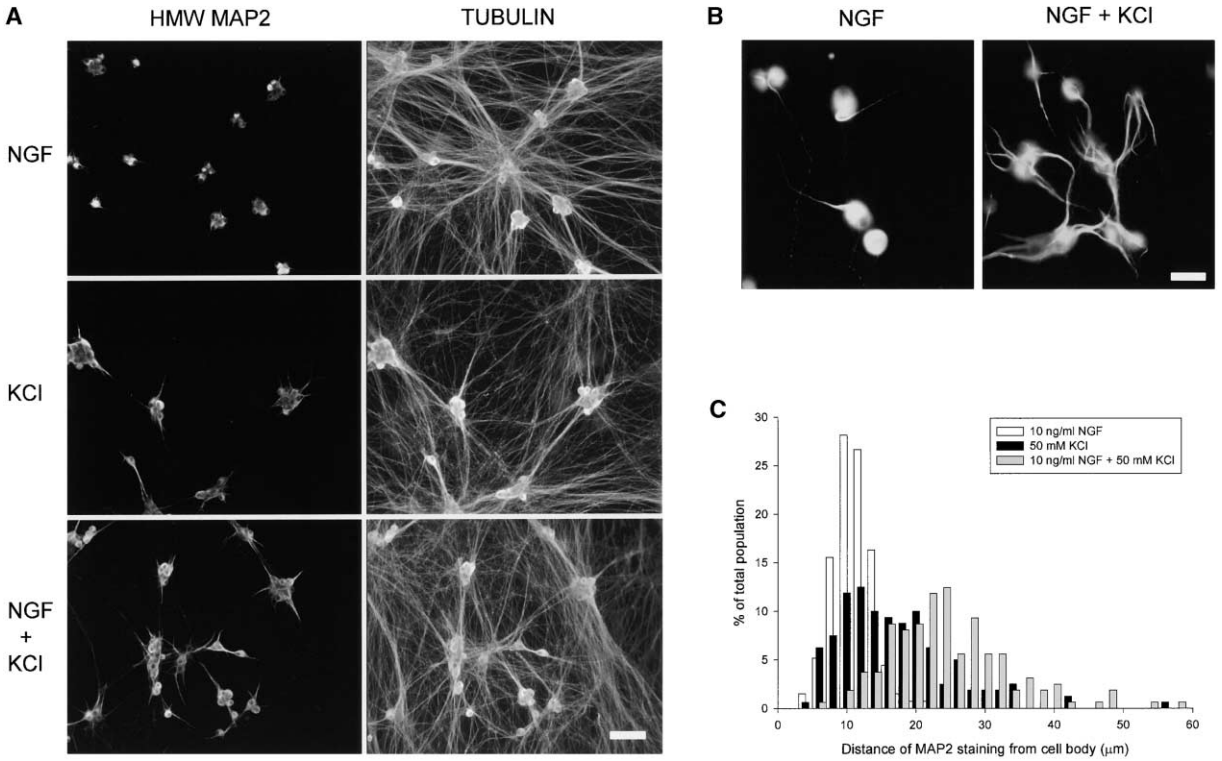

Figure 1. Neuronal Depolarization Causes Dendrite Formation in Cultured Sympathetic Neurons, and NGF Enhances This Dendritogenesis

(A) Double-label immunocytochemical analysis for HMW-MAP2 (left panels) and tubulin (right panels) in cultured sympathetic neurons that were grown for 5 days in 50 ng/ml NGF and then were switched for an additional 3 days into 10 ng/ml NGF (top panels), 50 mM KCl (middle panels), or 10 ng/ml NGF plus 50 mM KCl (bottom panels). Similar results were obtained in six independent experiments. Scale bar, 50 μM.

(B) Higher magnification of HMW-MAP2 staining in neurons cultured for 5 days in 50 ng/ml NGF and then switched for 3 days to 10 ng/ml NGF plus or minus 50 mM KCl. Scale bar, 10 μM.

(C) Quantification of the length of MAP2-positive processes in cultures similar to those shown in (A) and (B). Lengths of individual dendrites were quantified using image analysis, the data were normalized so that the total number of dendrites counted was equal to 100%, and the histograms were then plotted using bin sizes of 2 μM. Results derive from one representative experiment of five that were quantified, all of which produced similar results.

and hence dendritic stability, providing one potential structural mechanism underlying the effects of neurotrophins and neuronal activity on the formation and maintenance of neural circuitry.

Results

Neuronal Depolarization Causes Reversible Dendrite Formation in Cultured Sympathetic Neurons, and NGF Enhances This Dendritogenesis

Although NGF promotes survival and axonal growth in cultured neonatal sympathetic neurons, additional extrinsic cues are required for dendrite formation. To test the hypothesis that one additional requisite cue might be neuronal activity, neonatal sympathetic neurons were established for 5 days in 50 ng/ml NGF and were then switched into 50 mM KCl, which causes neuronal depolarization and is sufficient to maintain survival (Franklin et al., 1995; Vaillant et al., 1999). 3 days later, we performed double-label immunocytochemistry for HMW MAP2, a microtubule-associated protein that is dendrite specific (Bernhardt and Matus, 1984; Caceres et al., 1984), and for α-tubulin, which is present in both axons and dendrites (Figures 1A and 1B). This analysis revealed that, while axonal growth was very robust, dendrites were rudimentary or nonexistent on neurons cultured in NGF alone. In contrast, when neurons were switched into KCl for 3 days, they exhibited thick, MAP2-positive processes with the morphology of dendrites. Interestingly, when neurons were cultured in both KCl and NGF, NGF enhanced the length of the newly formed dendrites (Figures 1A and 1B).

To quantify this effect, random MAP2-labeled images were obtained from each of the treatments, and the length of all MAP2-positive processes in the images was determined. These measurements (which numbered 200–400 per treatment), were analyzed by sorting them into bins of 2 μM in size and normalizing the binning values to percentage of the total treatment population (Figure 1C). The resultant histograms confirmed that KCl caused a robust increase in the length of MAP2-positive processes and that NGF enhanced the length of these processes. This increase was statistically significant and reproducible. For example, in two representative experiments (of five experiments that were quantitated), neurons maintained in 10 ng/ml NGF alone had mean MAP2-positive process lengths in μm of 13.10 ± 0.49 and 13.87 ± 0.80, and sister cultures maintained in NGF plus KCl had mean lengths of 22.89 ± 0.84 and 21.95 ± 0.64, respectively ($p < 0.01$ in both cases).

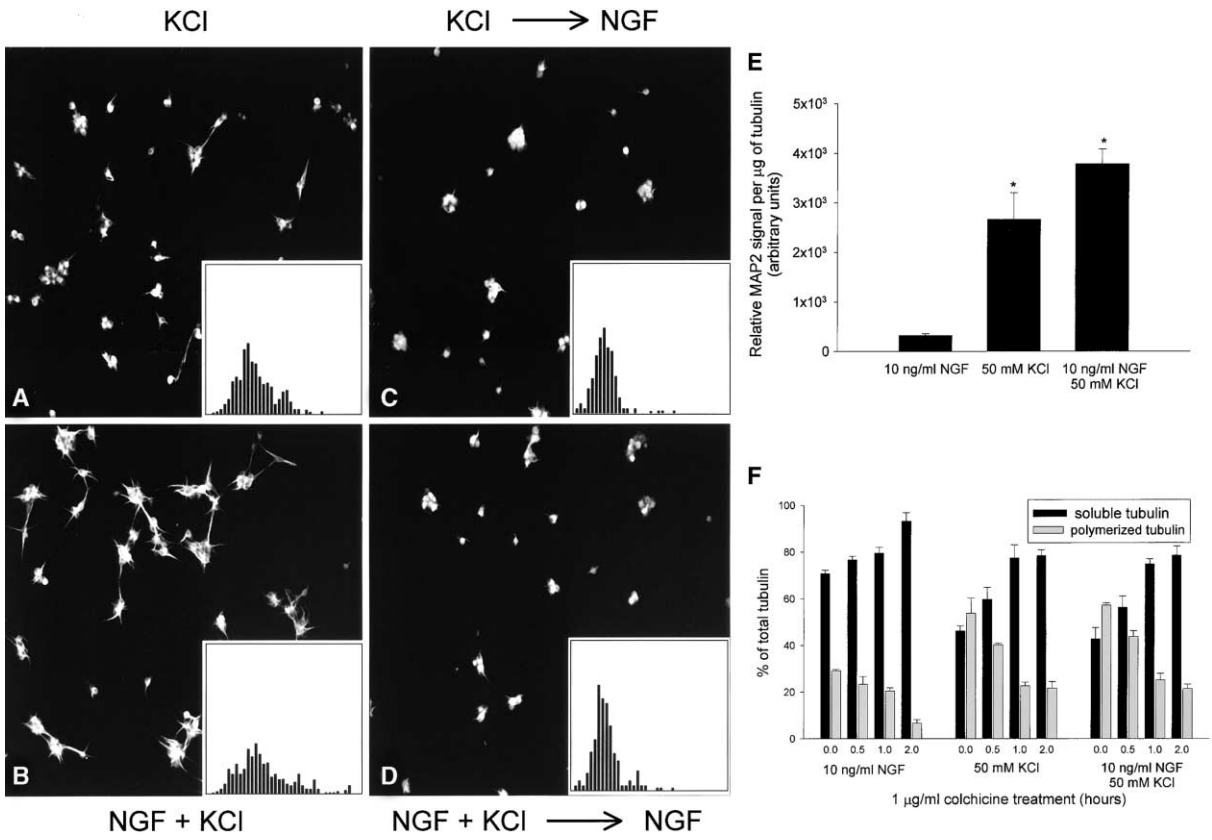

Figure 2. Activity-Dependent Dendrite Formation Is Reversible and Alters MAP2:Microtubule Dynamics

(A–D) Activity-dependent dendrite formation is reversible. Sympathetic neurons were cultured for 5 days in NGF and were then switched for 3 days into 50 mM KCl (A) or 10 ng/ml NGF plus 50 mM KCl (B). Both groups of neurons were then switched for a final 2 days into 10 ng/ml NGF alone (C and D). Cultures were immunostained for HMW-MAP2 (fluorescence micrographs), and morphometric analysis was performed as in Figure 1 (insets). The axes for the histograms are the same as in Figure 1C. Results derive from one representative experiment of three. A switch from NGF plus KCl into NGF alone produced a statistically significant decrease ($p < 0.01$), and dendrite lengths were not statistically different between neurons in NGF alone versus those that were switched from NGF plus KCl into NGF ($p > 0.05$).

(E and F) MAP2:microtubule dynamics in sympathetic neurons in response to NGF and KCl. (E) Quantification of the relative levels of MAP2 associated with assembled tubulin in sympathetic neurons established for 5 days in NGF, and then switched for 3 days into 10 ng/ml NGF, 50 mM KCl, or 10 ng/ml NGF plus 50 mM KCl. Assembled tubulin was isolated from the cultured neurons, and the relative amount of MAP2 present per μg of assembled tubulin was determined by quantitative immunoblot analysis. *$p < 0.01$ relative to 10 ng/ml NGF. (F) Percentage of soluble (disassembled) versus polymerized (assembled) tubulin in sympathetic neurons established in NGF, and then switched for 3 days into 10 ng/ml NGF, 50 mM KCl, or NGF plus KCl. Prior to lysis and analysis, cells were treated with colchicine for times ranging from 0.0 to 2.0 hr.

To test whether dendrite maintenance required constant exposure to KCl, neurons were established in NGF for 5 days, switched to KCl or KCl plus NGF for 3 days to induce dendrite formation, and then switched for an additional 2 days into NGF alone. Immunocytochemistry and image analysis revealed that the maintenance of MAP2-positive dendrites required the continued presence of KCl (Figures 2A–2D); neurons that were switched into NGF alone retracted their MAP2-positive processes so that the distribution histograms were statistically similar ($p > 0.05$) to those of neurons that had never seen KCl (Figures 2A–2D).

Depolarization Promotes the Association of Dendrite-Specific MAP2 with Microtubules and Enhances Microtubule Stability

The association of HMW MAP2 with microtubules enhances their stability and is thereby thought to play a key role in dendrite formation and maintenance (Gamblin et al., 1996; LeClerc et al., 1993; Caceres et al., 1984). To determine whether KCl caused the association of MAP2 with microtubules, we isolated assembled tubulin from sympathetic neurons cultured in KCl or KCl plus NGF, and then we quantified the relative amount of MAP2 associated with these microtubules by quantitative immunoblots (Vaillant et al., 1998). This analysis revealed that when neurons were maintained in NGF alone, relatively little MAP2 was associated with the assembled microtubules, and that treatment with KCl caused a 7-fold increase in this association (Figure 2E). The association of MAP2 with assembled microtubules was further potentiated by coincident treatment with NGF (Figure 2E).

We next determined the effect of KCl on microtubule stability by measuring the pool of assembled versus disassembled tubulin before and after treatment with the drug colchicine, which disassembles microtubules (Figure 2F). When neurons were maintained in NGF

alone, a relatively small fraction (28%) of the tubulin was assembled, and this pool was almost completely disassembled following exposure to 1 μg/ml colchicine for 2 hr (Figure 2F). In contrast, when neurons were cultured in KCl plus or minus NGF, 58% of the tubulin was in the assembled state initially, and even after 2 hr of colchicine treatment 24% of the tubulin was still assembled (Figure 2F). Since this latter assay measures tubulin in both axons and dendrites, and since the majority of processes in these cultures are axons (see Figure 1A), then these data indicate that KCl has a very significant effect in enhancing microtubule stability in both axons and dendrites, likely via MAP2 in dendrites, and potentially via axon-specific MAPs such as tau in axons (Caceres and Kosik, 1990).

Depolarization Causes Activation of CaMKII, a MAP2 Kinase, in Dendrites of Sympathetic Neurons

These data demonstrated that neuronal depolarization caused an increase in MAP2 association with microtubules and an increase in microtubule stability. One mechanism known to regulate the association of MAP2 with microtubules is phosphorylation (Sanchez et al., 2000). To determine whether depolarization regulated MAP2 phosphorylation, sympathetic neurons were exposed to KCl, MAP2 was immunoprecipitated, and the precipitated MAP2 was then analyzed for phosphorylation using an antibody specific for phosphoserine and phosphothreonine residues. These data demonstrated that KCl induced a robust phosphorylation of MAP2 (Figure 3A). Two kinases known to phosphorylate MAP2 on Ser/Thr residues in the microtubule binding domain are ERKs 1 and 2 (Ray and Sturgill, 1987; Sanchez et al., 2000) and CaMKII (Schulman, 1984), both of which are activated via neuronal depolarization (Vaillant et al., 1999). As a first step in determining whether these two kinases might be involved in the activity-dependent dendritogenesis seen here, we characterized long-term ERK and CaMKII activation. Sympathetic neurons were grown in 10 ng/ml NGF for 5 days and then switched into NGF, KCl, or KCl plus NGF for an additional 3 days. Western blot analysis with antibodies that recognize the phosphorylated, activated state of either CaMKII or the ERKs revealed that phosphorylated CaMKII was observed in all of the treatments, with higher levels in KCl with or without NGF versus NGF alone (Figure 3B). In contrast, as we have previously published (Vaillant et al., 1999), phosphorylated ERK levels were lower in KCl versus KCl plus NGF (Figure 3B). Reprobing of these same blots revealed that total ERK levels were constant in all treatments, and that, in agreement with the immunocytochemical data (Figure 1A), MAP2 levels were higher in KCl or KCl plus NGF than in NGF alone (Figure 3B).

We previously used in vitro kinase assays to demonstrate that the increased phospho-ERK observed following NGF or KCl treatment reflected an increase in ERK activation (Vaillant et al., 1999). We used a similar approach to confirm that the long-term increase in phospho-CaMKII noted here (Figure 3B) corresponded to increased activity. In agreement with the Western blots, kinase assays revealed that NGF led to a modest long-term activation of CaMKII, that KCl activated CaMKII to a greater degree than did NGF, and that the long-term activation was highest when neurons were exposed to both NGF and KCl (Figure 3C). Similar results were obtained when immunoprecipitations were performed with antibodies to the α (Figure 3C) or β subunit (data not shown) of CaMKII.

These data demonstrated that two kinases known to phosphorylate MAP2, CaMKII, and the ERKs are activated in a long-term fashion by KCl and NGF. Previous work has shown that the ERKs are activated in both cell bodies and neurites of sympathetic neurons in response to NGF (Atwal et al., 2000). We therefore determined where in the cells CaMKII was activated; to perform these studies, we performed immunocytochemistry using phospho-CaMKII. This analysis revealed that, in contrast to the ERKs, in NGF alone phospho-CaMKII immunostaining was limited to the cell bodies of neurons, with little or no detectable immunoreactivity in neurites (Figure 3D). However, when KCl was also present, phospho-CaMKII was present in cell bodies, dendrites, and in MAP2-negative neurites that were presumably axons. Strikingly, much of the phospho-CaMKII immunoreactivity appeared as very bright spots of staining along the length of neuronal processes and/or on cell bodies. A similar pattern of immunostaining was observed with KCl alone (data not shown). Thus, KCl, but not NGF, leads to detectable activation of CaMKII in axons and dendrites.

CaMKII and ERK Activation Are Essential for Depolarization-Dependent Dendrite Formation

To determine whether activation of CaMKII and/or the ERKs was important for KCl-mediated dendrite formation, we utilized a number of pharmacological inhibitors that are specific for one or the other pathway. Specifically, we utilized PD98059, which specifically inhibits MEK1 (Alessi et al., 1995), the kinase that activates ERKs 1 and 2, and KN-62 (Tokumitsu et al., 1990) and myristoylated autocamtide inhibitory peptide (mAIP) (Ishida et al., 1995), two agents that inhibit CamKII via different mechanisms. As controls, we utilized two drugs that perturb protein kinase A, H-89 (a PKA antagonist), and forskolin; PKA phosphorylates MAP2, but it does so outside of the microtubule binding domain (Rubino et al., 1989; Obar et al., 1989).

Initially, we confirmed the specificity of these drugs in sympathetic neurons. Neurons were selected in NGF and were then switched into NGF, KCl, or NGF plus KCl with or without one of the drugs. 15 min later, neurons were lysed, and we then performed either Western blots or activity assays. As a control, we examined Akt activation using a phosphorylation-specific antibody; Akt is a kinase that plays an essential role in survival of these neurons (reviewed in Kaplan and Miller, 2000) and that should not be affected by any of these pharmacological manipulations. Western blot analysis confirmed, as we have previously published (Vaillant et al., 1999), that both NGF and KCl cause increased levels of phospho-ERK and phospho-Akt (Figure 4G). As predicted, none of the five compounds that we tested had any effect on phospho-Akt in response to NGF plus KCl, and the only drug that affected phospho-ERK levels was PD98059, which completely blocked ERK activation (Figure 4G). Similarly, CaMKII assays revealed that both 10 μM

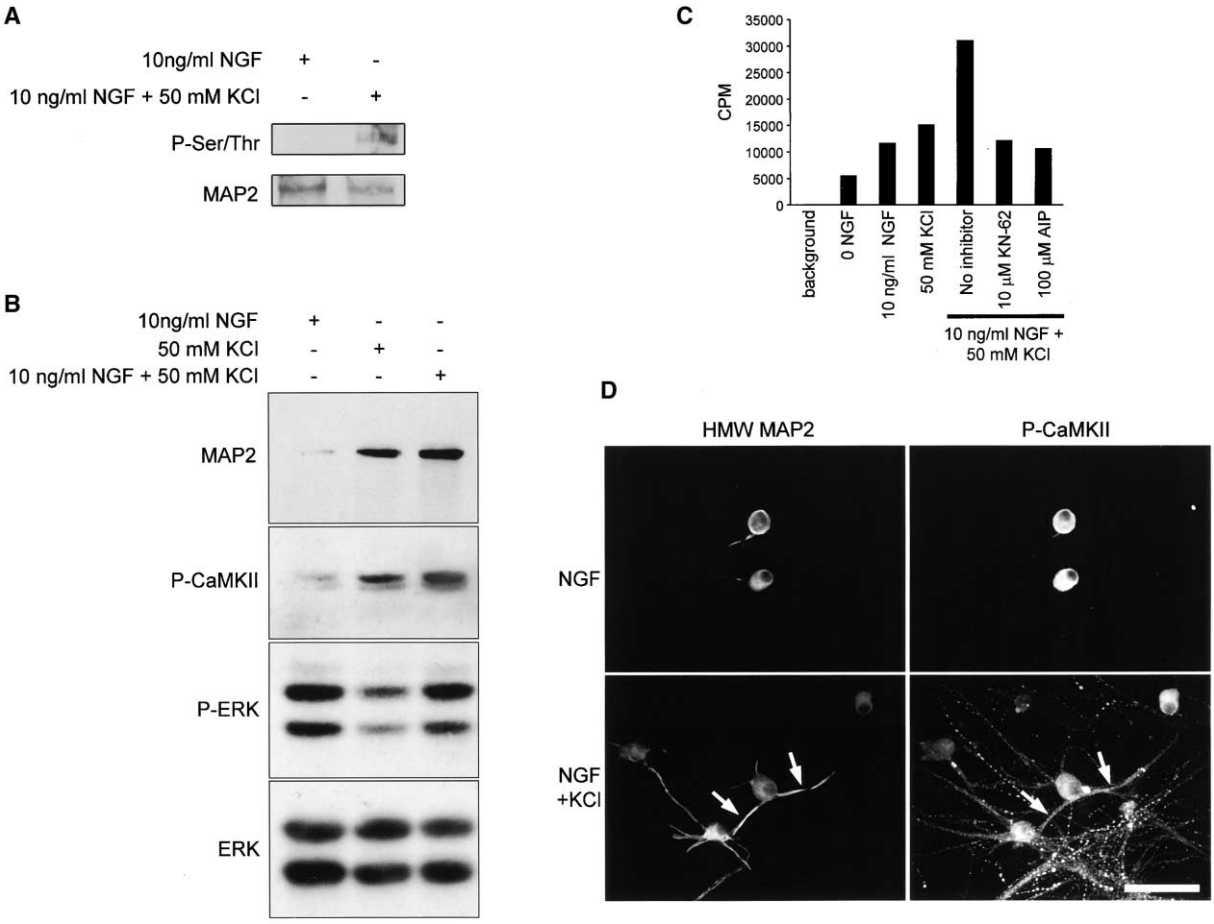

Figure 3. Depolarization and NGF Converge to Enhance MAP2 Levels and Promote Long-Term CaMKII and ERK Activation in Sympathetic Neurons

(A) Western blot analysis for serine/threonine phosphorylation of immunoprecipitated MAP2 (top panel) in neurons that were washed free of NGF and then induced with 10 ng/ml NGF or 10 ng/ml NGF plus 50 mM KCl. Western blot analysis of MAP2 in equal amounts of protein from the same neuronal lysates (bottom panel) revealed that levels of MAP2 were similar.

(B) Western blot analysis of sympathetic neurons established in NGF and then switched into NGF, KCl, or NGF plus KCl for 3 days. Equal amounts of protein from each sample were probed for MAP2, the phosphorylated state of CaMKII (P-CaMKII), phospho-ERK (P-ERK), or total ERK protein. Similar results were obtained in three independent experiments.

(C) In vitro kinase activity assay for CaMKII. Neurons were established in NGF, and then switched for 3 days into NGF, KCl, or NGF plus KCl. Some cultures were also treated with NGF plus KCl plus 10 μM KN-62 or 100 μM AIP, both CaMKII inhibitors. CaMKII was then immunoprecipitated from neuronal lysates using an antibody directed to the α subunit of CaMKII. CaMKII activity in the immunoprecipitate was measured by quantifying phosphotransferase activity on to a CaMKII-specific peptide, AIP. Similar results were obtained using a monoclonal antibody directed against the β subunit of CaMKII (data not shown).

(D) Depolarization leads to CaMKII phosphorylation in dendrites of sympathetic neurons. Double-label immunocytochemistry for HMW-MAP2 (left panels) and phospho-CaMKII (right panels; P-CaMKII) in neurons established in NGF and then switched for 3 days to 10 ng/ml NGF (NGF) or 10 ng/ml NGF plus 50 mM KCl (NGF + KCl). Note that in cells treated with NGF plus KCl, punctate P-CaMKII staining can be seen in processes that colocalize with HMW-MAP2 (arrows). Similar results were obtained in four independent experiments. Scale bar, 30 μM.

KN-62 and 100 μM mAIP inhibited CaMKII activation seen in NGF plus KCl (Figure 3C), with no effect on phospho-Akt or phospho-Erk levels (Figure 4G).

We next confirmed that these pharmacological treatments had no unanticipated effects on neuronal survival; we previously showed that Akt, but not the ERKs, is required for survival in NGF or NGF plus KCl and that CaMKII is important for survival only when neurons are maintained in KCl alone (Vaillant et al., 1999). Neurons were selected in NGF and then switched for 2 days into NGF and/or KCl plus or minus the same drug concentrations as were used for the biochemistry. MTT assays (Figure 4H) confirmed that inhibition of the MEK-ERK pathway or of CamKII had no effect on survival in NGF or in NGF plus KCl and that inhibition of CaMKII with either KN-62 or mAIP completely inhibited neuronal survival in the presence of KCl alone.

Having performed these controls, we next asked whether inhibition of either the MEK-ERK pathway or of CaMKII affected KCl-mediated dendrite formation. Neurons were selected in NGF and then were switched to NGF plus KCl with or without the various inhibitors for 3 days. Immunocytochemistry and image analysis revealed that inhibition of either the MEK-ERK pathway with PD98059 or CaMKII with KN-62 or mAIP was sufficient to completely block activity-dependent dendrite

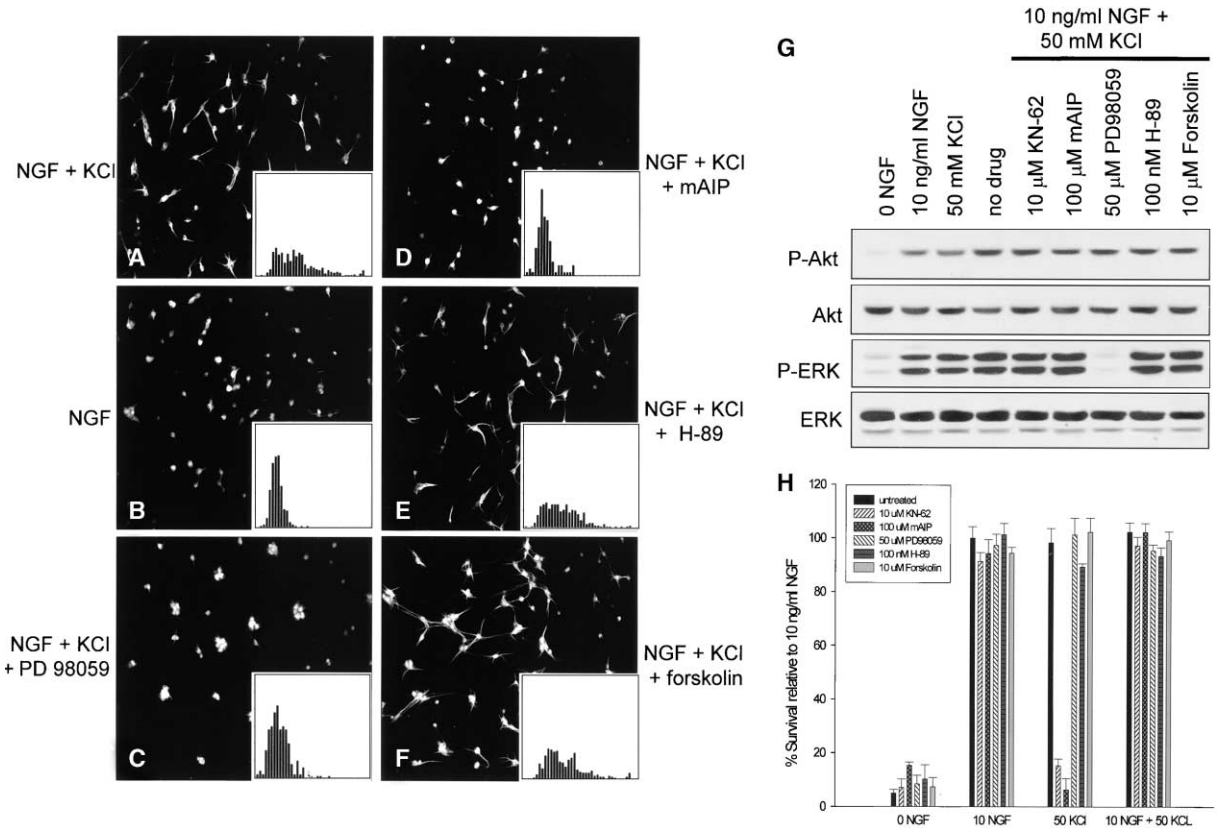

Figure 4. Inhibition of Either CaMKII or the MEK-ERK Pathway Is Sufficient to Inhibit Activity-Dependent Dendrite Formation

(A–F) Inhibition of either CaMKII or the MEK-ERK pathway is sufficient to inhibit activity-dependent dendrite formation. Immunofluorescence microscopy for HMW MAP2 in sympathetic neurons established in NGF, and then switched for 3 days to 10 ng/ml NGF plus 50 mM KCl (A) with various pharmacological inhibitors (C–F). In (B), neurons were switched into 10 ng/ml NGF alone. The pharmacological inhibitors used were (C) 50 μM PD98059, (D) 100 μM mAIP, (E) 100 nM H-89, or (F) 10 μM forskolin. The insets depict morphometric analysis of dendrites as described in Figure 1. Axes of the histograms are as in Figure 1C. Note that inhibition of CaMKII with mAIP or of MEK with PD98059 inhibited dendrite formation. Similar results were obtained in three independent experiments.

(G) Specificity of pharmacological reagents. Western blot analysis of lysates of sympathetic neurons established for 5 days in NGF, and then switched for 15 min into NGF, KCl, or NGF plus KCl with and without the CaMKII inhibitors 10 μM KN-62 and 100 μM mAIP, the MEK inhibitor 50 μM PD98059, the PKA inhibitor 100 nM H-89, or 10 μM forskolin. Equal amounts of protein were probed with antibodies specific to phosphorylated Akt (P-Akt) or phosphorylated ERKs (P-ERK) and then reprobed with antibodies to total Akt or ERKs.

(H) Neuronal survival mediated by KCl, but not KCl plus NGF, requires CaMKII. MTT survival assays of sympathetic neurons established in NGF, and then switched for 2 days into NGF, KCl, or NGF plus KCl with or without the same pharmacological inhibitors as used in (A).

formation ($p < 0.01$) (Figures 4A–4D). Moreover, neither H-89 or forskolin had any effect (Figures 4E and 4F), indicating the specificity of this inhibition.

To confirm that this inhibition of MAP2-positive dendrites was accompanied by biochemical alterations in MAP2:microtubule interactions, we first measured the levels of MAP2 present in sympathetic neurons in the presence of NGF plus KCl with and without the various drugs. Western blots revealed that PD98059, KN-62, and mAIP all inhibited the accumulation of MAP2 that occurred when neurons were cultured in the presence of NGF plus KCl (Figure 5A). We next measured the relative level of MAP2 that was associated with assembled microtubules under the same conditions (Figure 5B). Isolation of the assembled microtubule fraction followed by quantitative dot blots with anti-MAP2 indicated that PD980569 and KN-62 completely inhibited the MAP2:microtubule association that was induced by KCl plus NGF (Figure 5B). Finally, we examined the proportion of assembled tubulin in the presence or absence of colchicine. This analysis revealed that, as seen for the MAP2 measurements, the large increase in the proportion of assembled microtubules that was induced by KCl plus NGF was completely inhibited by PD98059 or KN-62 (Figure 5C). In contrast, treatment with H-89 or forskolin had no effect on any of these biochemical measurements (Figures 5A–5C). Thus, the inhibition of dendrite formation that occurs following inhibition of the MEK-ERK pathway or of CaMKII is likely mediated directly via regulation of MAP2:microtubule interactions.

Once Dendrites Are Formed, Both CaMKII and the ERKs Must Be Inhibited to Cause Them to Retract

Since withdrawal of KCl is sufficient to cause sympathetic neurons to retract their dendrites (Figures 2A–2D), we next asked whether inhibition of either CaMKII or the MEK-ERK pathway was also sufficient for retraction. To perform these experiments, sympathetic neurons

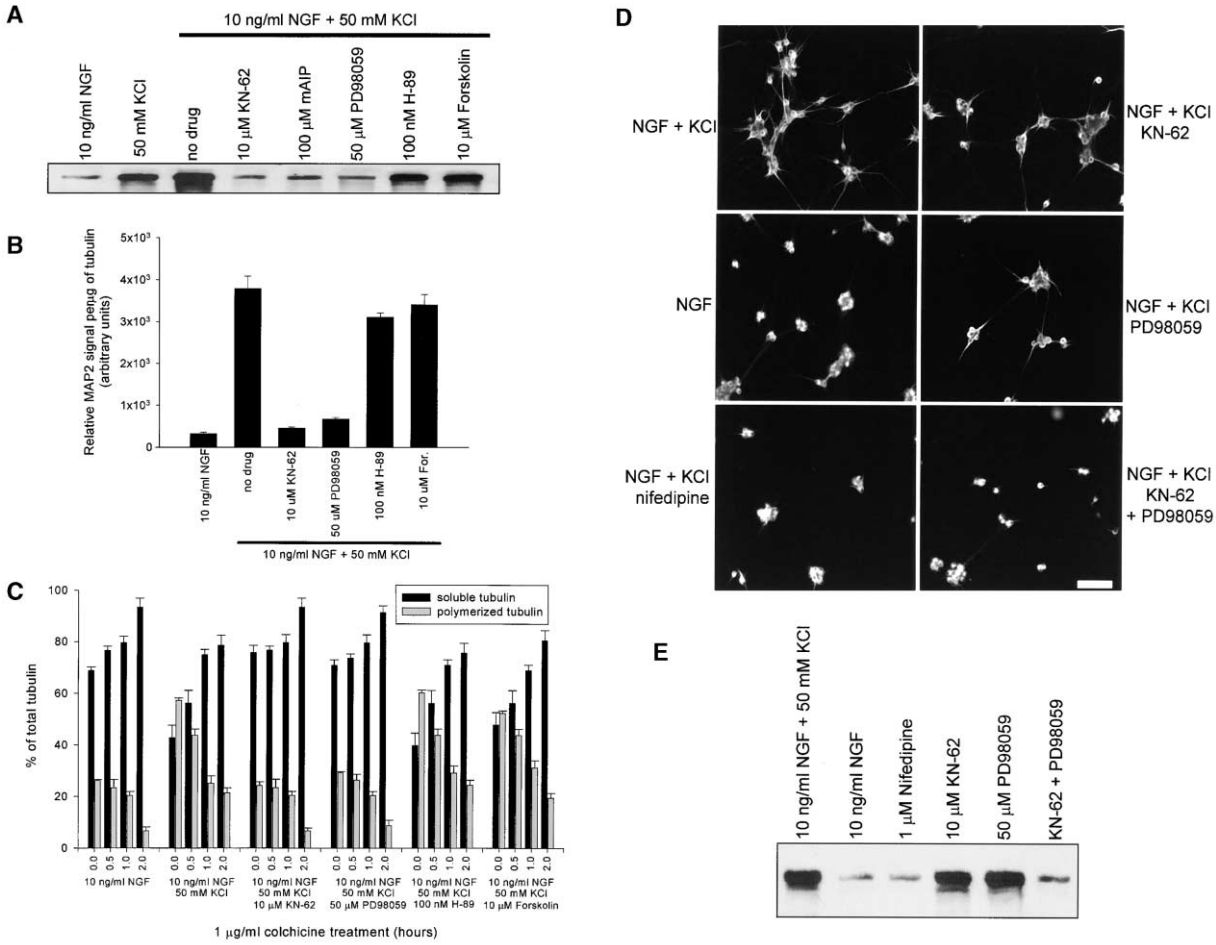

Figure 5. CaMKII and MEK Regulate MAP2:Microtubule Dynamics and Dendrite Retraction

(A–C) Inhibition of either CaMKII or MEK blocks the activity-dependent accumulation of MAP2, association of MAP2 with microtubules, and subsequent stabilization of microtubules. (A) Western blot analysis of HMW-MAP2 in lysates of sympathetic neurons established in NGF, and then switched for 3 days into NGF, KCl, or NGF plus KCl with and without the same inhibitors as described in Figure 5. (B) Quantification of the relative amount of HMW-MAP2 associated with assembled tubulin in sympathetic neurons treated as in (A). Quantitative immunoblots were performed as in Figure 2. (C) Percentage of soluble (disassembled) versus polymerized (assembled) tubulin in sympathetic neurons treated as in (A). Cells were treated with colchicine with periods of time ranging from 0.0 to 2.0 hr prior to lysis and biochemical analysis.

(D and E) Concurrent inhibition of CaMKII and MEK is sufficient to cause retraction of previously-formed dendrites.

(D) Immunofluorescence microscopy of HMW-MAP2 in sympathetic neurons established in NGF for 5 days, switched for 3 days to NGF plus KCl to cause dendrite formation, and then switched for an additional 2 days into 10 ng/ml NGF alone (middle left panel) or into 10 ng/ml NGF plus 50 mM KCl with (top left panel) or without various pharmacological inhibitors. The inhibitors used were 1 μM nifedipine, which blocks L-type calcium channels, 10 μM KN-62, 50 μM PD98059, or 10 μM KN-62 plus 50 uM PD98059. Similar results were obtained in two independent experiments. Scale bar, 100 μM.

(E) Western blot analysis for HMW-MAP2 in lysates of sympathetic neurons treated as in (A).

were selected in NGF for 5 days, switched to NGF plus KCl for 3 days to cause dendrites to form, and then were switched to NGF plus KCl with or without PD98059 and/or KN-62. Immunocytochemistry and image analysis revealed that while a switch into NGF alone was sufficient to cause retraction, neither KN-62 or PD98059 had any effect on the dendrites that were already formed (Figure 5D). However, when both the MEK-ERK pathway and CaMKII were inhibited by using both drugs together, dendrites significantly retracted ($p < 0.01$), a result that was also obtained when KCl-mediated Ca^{2+} influx via L-type calcium channels was inhibited using the drug nifedipine (Figure 5D). Biochemical confirmation of these results was obtained by performing Western blot analysis for MAP2 (Figure 5E), which demonstrated that only nifedipine and KN-62 plus PD98059, but neither of these latter two drugs alone, inhibited the KCl-induced increase in MAP2 levels.

Field Stimulation and Cholinergic Receptor Activation Are Both Sufficient to Cause Reversible Dendrite Formation

To determine whether the results obtained here with KCl-mediated depolarization generalized to other stimuli that promoted neuronal activity, we performed experiments with field stimulation and, because sympathetic neurons receive cholinergic inputs in vivo, with the cholinergic receptor agonist carbachol. For the field stimula-

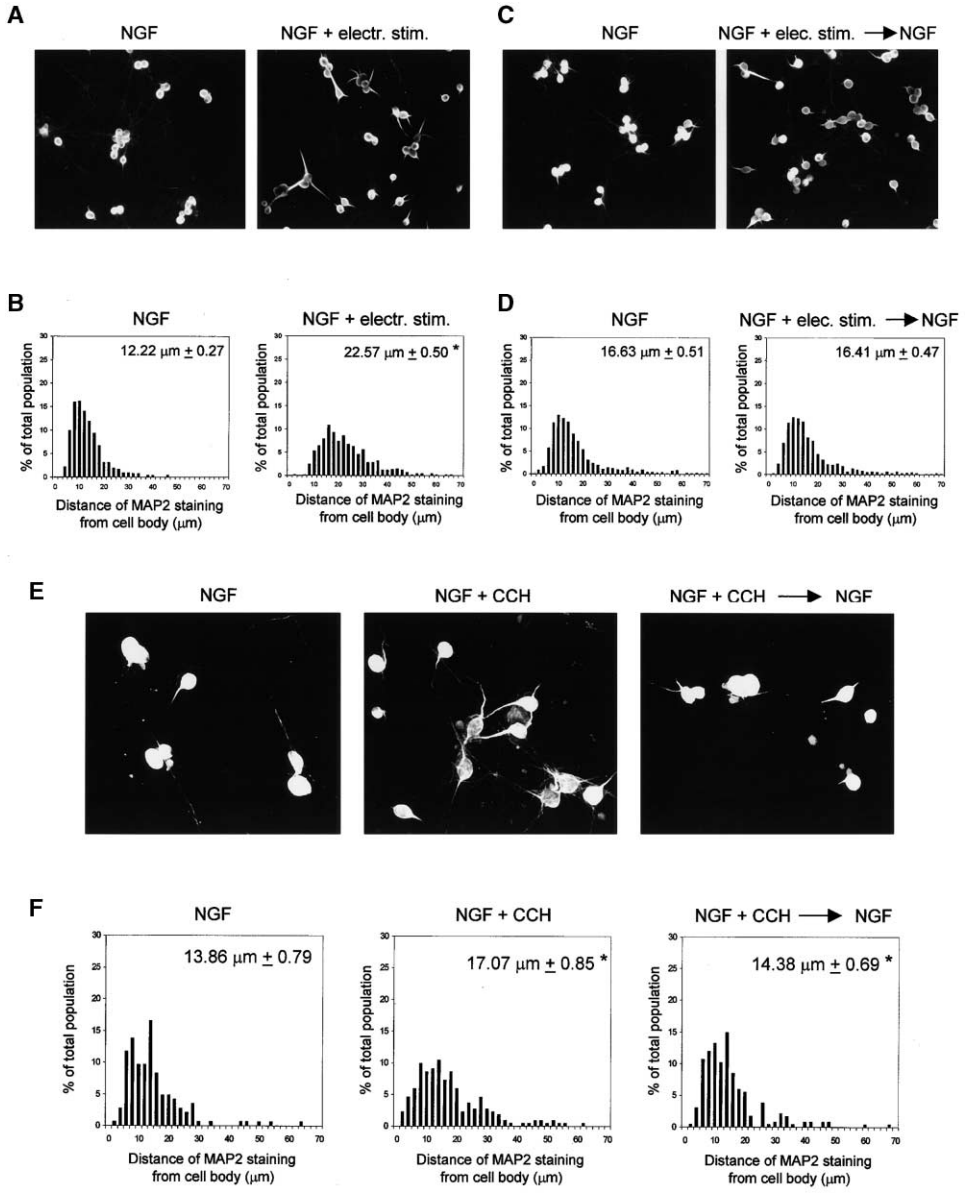

Figure 6. Field Stimulation and Acetylcholine Receptor Activation Both Mediate Reversible Dendrite Formation in Sympathetic Neurons

(A) Immunocytochemical analysis for HMW-MAP2 in cultured sympathetic neurons switched into 10 ng/ml NGF and stimulated (right panel) or not (left panel) for 3 days.

(B) Quantification of the length of MAP2-positive processes in cultures similar to those shown in (A). Results are indicated both as a distribution histogram and as a mean dendrite length ± SE. *$p < 0.01$.

(C) Immunocytochemical analysis for HMW-MAP2 in neurons switched into 10 ng/ml NGF for 5 days with no stimulation (left panel) or stimulated for 3 days and then left an additional 2 days with no stimulation (right panel).

(D) Quantification of the length of MAP2-positive processes in cultures similar to those shown in (C).

(E) Immunocytochemical analysis for HMW-MAP2 in neurons either switched into 10 ng/ml NGF for 5 days (left panel), switched into NGF plus 100 μM carbachol for 5 days (center panel), or switched into NGF plus carbachol for 3 days and then switched back into 10 ng/ml NGF for a final 2 days (right panel).

(F) Quantification of the length of MAP2-positive processes in cultures similar to those shown in (E). For NGF + CCH, *$p < 0.01$ relative to NGF alone. For NGF + CCH switched into NGF alone, *$p < 0.01$ relative to neurons maintained in NGF + CCH for the entirety of the experiment.

tion experiments, neurons were established for 5 days and then were switched to 10 ng/ml NGF and stimulated for 3 days with 0.2 ms pulses of alternating polarity delivered at 5 Hz. Immunocytochemistry of these stimulated neurons for MAP2 revealed that, like KCl depolarization, patterned field stimulation robustly promoted dendrite formation (Figure 6A). Image analysis and binning of dendrite lengths showed a large, statistically significant shift in size of MAP2-positive dendrites in neurons that were stimulated relative to unstimulated neurons cultured in the same multiwell dish (Figure 6B). In three independent experiments, the mean MAP2-positive process length in μm of unstimulated neurons was 12.22 ± 0.27, 11.08 ± 0.48, and 13.69 ± 0.48, while

the mean dendrite lengths for stimulated sister cultures were 22.57 ± 0.50, 20.91 ± 0.58, and 22.27 ± 0.54, respectively ($p < 0.01$ in all cases).

To ask whether this activity-dependent dendrite formation was reversible, neurons were established for 5 days, stimulated for 3 days, and then were left unstimulated for an additional 2 days. Immunocytochemistry and image analysis (Figures 6C and 6D) revealed that, as seen for KCl-mediated depolarization, 2 days without electrical stimulation caused dendritic retraction.

We next asked whether cholinergic receptor activation could promote dendrite formation. For these experiments, neurons were established in NGF for 5 days, and then were switched into 10 ng/ml NGF plus or minus 100 μM carbachol for 3 days. Immunostaining with anti-MAP2 revealed that carbachol stimulated dendrite formation ($p < 0.01$), but to a lesser degree than did KCl or field stimulation (Figure 6E). To determine whether this carbachol-dependent dendrite formation was also reversible, neurons were treated with carbachol plus NGF for 3 days and then switched into NGF alone for an additional 2 days. Immunocytochemistry and image analysis revealed that dendritic maintenance required the constant presence of carbachol (Figures 6E and 6F). Thus, three different stimuli that mediate neuronal activity, field stimulation, cholinergic receptor activation, and KCl-mediated depolarization all reversibly promote sympathetic neuron dendrite formation.

Field Stimulation and Cholinergic Receptor Activation Both Require ERK and CamKII Activity to Promote Dendrite Formation

To ask whether the signaling pathways regulating activity- and carbachol-dependent dendrite formation were similar to those seen with KCl, we utilized KN62 to inhibit CaMKII and PD98059 to inhibit MEK. Immunocytochemical analysis of sympathetic neurons that were exposed to field stimulation for 3 days in NGF plus or minus one of these two inhibitors revealed that suppression of either CaMKII or MEK activity was sufficient to inhibit dendrite formation (Figures 7A and 7B). Dendrite length distribution histograms and mean dendrite length were essentially the same for neurons stimulated in the presence of KN62 and those that were unstimulated (Figure 7A). Treatment with PD98059 was only slightly less effective, inhibiting the activity-dependent increase in mean dendrite length by 71% (Figure 7B).

Similar results were obtained for carbachol-dependent dendrite formation. While neurons treated with NGF plus carbachol for 3 days had a mean dendrite length of 16.27 μm ± 0.58, those treated with carbachol plus PD98059 and KN62 had mean dendrite lengths of only 11.13 ± 0.41 and 13.14 ± 0.52 μm (Figure 7C), measurements similar to those seen with NGF alone (Figure 6F). Biochemical measurements of the relative amount of MAP2 associated with assembled microtubules confirmed this conclusion. Carbachol enhanced the amount of MAP2 associated with microtubules, but treatment with either PD98059 or KN62 was sufficient to inhibit this carbachol-dependent increase (Figure 7D). Thus, activity-dependent dendrite formation requires ERK and CaMKII activation, regardless of the means of neuronal stimulation.

Discussion

Together, these data support a number of major conclusions. First, our findings show that neuronal activity, as mediated by field stimulation, by cholinergic receptor activation, or by KCl, causes sympathetic neuron dendrite formation. In addition, while NGF on its own does not support dendrite formation, it enhances this activity-dependent dendritogenesis. Second, data presented here demonstrate that this activity-dependent dendrite formation is reversible; withdrawal of any of the three stimuli used here caused dendrites to retract completely. Thus, ongoing activity is required not just for formation of dendrites, but also for their maintenance. Third, our results demonstrate that activity-dependent dendrite formation is ultimately mediated at the cytoskeletal level; depolarization results in increased MAP2 levels, increased MAP2 phosphorylation, increased association of MAP2 with microtubules, and a subsequent increase in microtubule stability. Fourth, these activity-driven effects on the cytoskeleton and on dendrite formation require activation of two kinases that phosphorylate MAP2 in the microtubule binding domain, CaMKII and the ERKs. Inhibition of depolarization-dependent activation of either kinase is sufficient to inhibit dendritogenesis, while inhibition of both together caused dendrites to retract. Together, these data indicate that ongoing neuronal activity is essential for the formation and maintenance of dendrites, a finding that has broad implications for development and for activity-dependent plasticity. Moreover, our findings support a model where neurotrophins and depolarization converge intracellularly to regulate dendritic morphology (Figure 7E), a model that involves kinases, the ERKs and CaMKII, which are known to play essential roles in neural plasticity (Braun and Schulman, 1995; Impey et al., 1999).

These findings have implications both for neuronal development and for plasticity in the mature nervous system. With regard to developing neurons, these data suggest that one trigger for dendritic growth is initial formation of an active circuit and that exposure to neurotrophins then potentiates activity-dependent dendritogenesis. For peripheral sympathetic neurons, such a model fits well with the in vivo data; sympathetic neurons have only a rudimentary dendritic arbor until they make contact with their target tissue (Rubin, 1985a, 1985b), and once such an initial circuit is established, the absolute size of the dendritic arbor is a function of the amount of target territory (and hence the amount of target-derived NGF) "seen" by any individual neuron (Voyvodic, 1989). Moreover, axotomy causes dendrites to retract until target contact is reestablished (Purves, 1975; Yawo, 1987), and addition of exogenous NGF causes dendritic arbors to expand (Snider, 1988; Ruit et al., 1990). Such a model does not imply that other extrinsic cues are unimportant for dendrite formation during development. In fact, BMP-7 (also called OP-1) has been shown to promote dendrite formation in cultured sympathetic neurons (Lein et al., 1995), and IGF-1 enhances growth of some developing cortical dendrites (Niblock et al., 2000).

Of equal interest to the developmental conclusions are the implications of our findings for structural plastic-

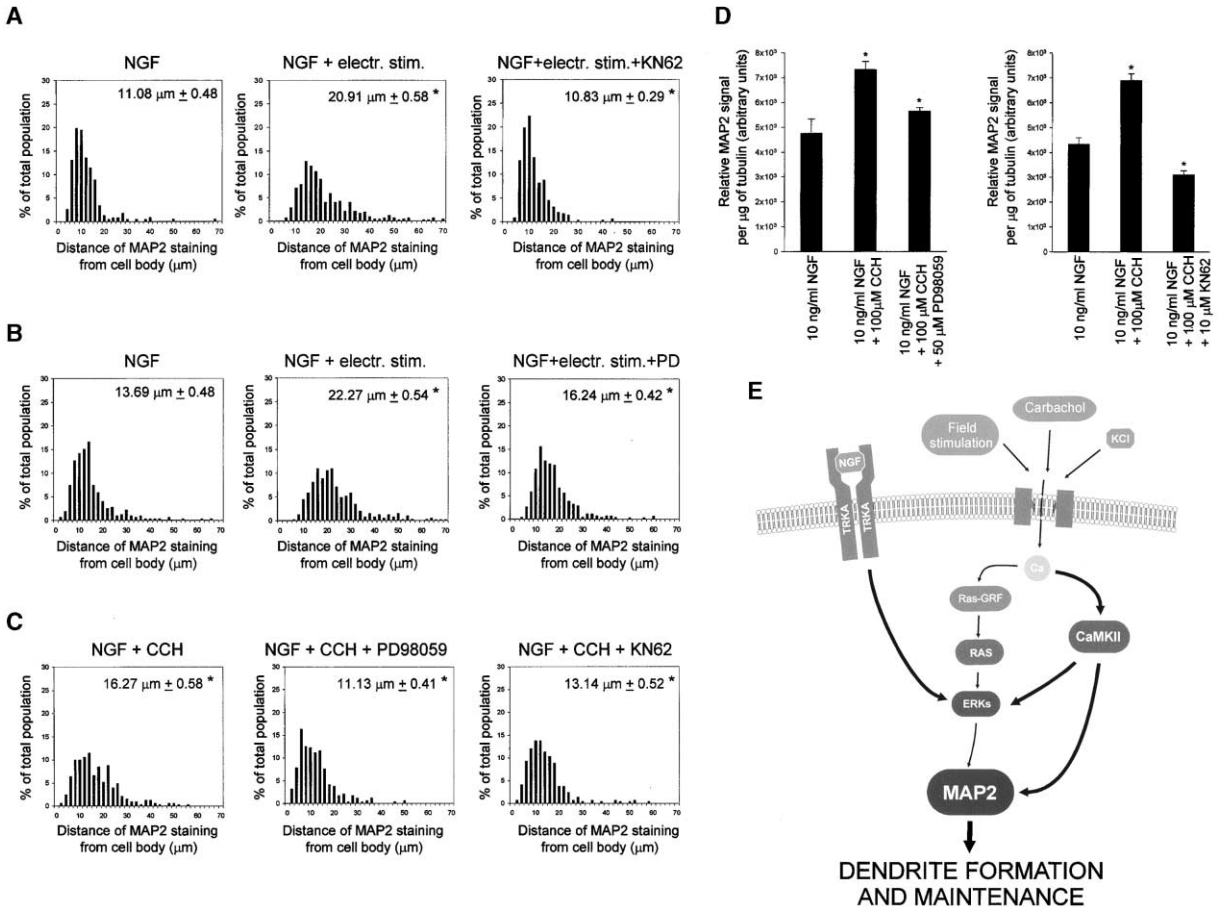

Figure 7. Dendrite Formation Induced by Field Stimulation or by Acetylcholine Receptor Activation Requires Coactivation of CaMKII and the MEK-ERK Pathway

(A) Quantification of the length of MAP2-positive processes in sympathetic neuron cultures that were switched into 10 ng/ml NGF for 3 days (left panel) or that were switched into 10 ng/ml NGF and stimulated for 3 days in the presence (right panel) or absence (middle panel) of 10 μM KN62. Results are indicated both as a distribution histogram and as mean dendrite length ± SE. For NGF + electr. stim. (electrical stimulation), *$p < 0.01$ relative to NGF alone. For NGF + electr. stim. + KN62, *$p < 0.01$ relative to NGF + electr. stim.

(B) Quantification of MAP2-positive processes in neurons that were maintained in 10 ng/ml NGF (left panel) or stimulated for 3 days in 10 ng/ml NGF with (right panel) or without (middle panel) 50 μM PD98059. For NGF + electr. stim., *$p < 0.01$ relative to NGF alone, while for NGF + electr. stim. + PD, *$p < 0.01$ relative to NGF + electr. stim.

(C) Quantification of MAP2-positive processes in neurons cultured for 3 days in 10 ng/ml NGF plus 100 μM carbachol without (left panel) or with 10 μM KN62 (middle panel) or 50 μM PD98059 (right panel). For NGF + CCH, *$p < 0.01$ relative to NGF alone for the same experiment; for NGF + CCH + PD98059, *$p < 0.01$ relative to NGF + CCH; while for NGF + CCH + KN62, *$p < 0.01$ relative to NGF + CCH.

(D) Quantification of the relative amount of HMW-MAP2 associated with assembled tubulin in sympathetic neurons treated as in (C). *$p < 0.05$.

(E) Proposed model for signal transduction mechanisms involved in activity-dependent dendrite formation. In this model, both the ERKs and CaMKII must be activated for enhancement of MAP2:microtubule interactions and subsequent microtubule stabilization. The continued MAP2:microtubule association requires the ongoing activation of both of these pathways in dendrites. Increased calcium, which causes activation of both ERKs and CaMKII, is required for dendrite formation, while NGF-mediated activation of TrkA collaborates with the calcium influx at the level of ERK and CaMKII activation and, potentially, via enhanced synthesis of HMW-MAP2.

ity in mature neurons. Live imaging of adult sympathetic neurons in vivo has shown that sympathetic dendrites are dynamic (Purves et al., 1986). Since data here indicate that activity promotes dendritic growth, then we propose that if a sympathetic neuron is particularly active, its dendrites would expand, and the resultant larger dendritic arbor would secrete increased levels of a retrograde neurotrophic factor to promote the formation of new preganglionic synapses (Purves, 1988). In this regard, we have previously demonstrated that the neurotrophin BDNF (Leibrock et al., 1989) is one such sympathetic neuron-derived retrograde factor—sympathetic neurons make BDNF (Causing et al., 1997), BDNF is localized to sympathetic neuron dendrites (C. Causing and F.D.M., unpublished data), and increased sympathetic neuron-derived BDNF enhanced the number of preganglionic:sympathetic neuron synapses (Causing et al., 1997). This type of activity- and neurotrophin-dependent model would thus provide a feed-forward mechanism for strengthening active preganglionic neuron:sympathetic neuron:target tissue connections.

How does neuronal activity lead to the formation and/

or maintenance of dendrites? Data presented here indicate that three different stimuli that promote sympathetic neuron dendritogenesis all require coactivation of CaMKII and the ERKs. With regard to KCl depolarization, we (Vaillant et al., 1999) have previously demonstrated that in sympathetic neurons, depolarization causes long-term activation of the ERKs via Ras, and we demonstrate here that it also causes activation of CaMKII, as monitored by phosphorylation and by kinase assays. We propose that coincident activation of both of these pathways then causes the cytoskeletal alterations that we have documented here: increased levels and phosphorylation of HMW MAP2 (the dendrite-specific isoform), increased association of MAP2 with microtubules, and increased microtubule stability. Since MAP2 is a direct substrate of both CaMKII and the ERKs (Sanchez et al., 2000; Ray and Sturgill, 1987; Schulman, 1984), then these kinases might directly phosphorylate MAP2 and enhance its association with microtubules, thereby leading to increased microtubules stability (Jameson and Caplow, 1981; Brugg and Matus, 1991; Sanchez et al., 2000). A second possibility is that both of these pathways enhance MAP2 levels and, thereby, increase MAP2:microtubule interactions. These explanations are not mutually exclusive.

A number of intriguing papers indicate that CaMKII and the MEK-ERK pathway are also important for dendrite formation in central neurons. In developing *Xenopus* retinotectal neurons, CaMKII activity is required for dendritic maturation and stabilization (Wu and Cline, 1998), and in hippocampal neurons MEK-ERK activation has been shown to be essential for protrusion of new dendritic filopodia in response to local activity (Wu et al., 2001). Furthermore, in hippocampal neurons, depolarization or activation of metabotropic glutamate receptors caused phosphorylation of MAP2 via a mechanism requiring CaMKII or the ERKs (Díaz-Nido et al., 1993; Quinlan and Halpain, 1996). These findings, together with studies defining a role for CaMKII and the MEK-ERK pathway in at least some types of neural plasticity (Braun and Schulman, 1995; Impey et al., 1999), suggest that the convergent signaling mechanisms that we describe here for peripheral dendrites may well generalize to both developing and mature CNS neurons.

Data presented here also indicate that NGF-mediated TrkA activation is not sufficient to form or maintain dendrites, but that it can collaborate with neuronal activity to enhance dendritic growth. Since TrkA activates the ERKs in sympathetic neurons as well or better than 50 mM KCl (Vaillant et al., 1999; data shown here), then these findings suggest that the key "trigger" for activity-dependent dendritogenesis in these neurons is CaMKII. Although NGF does activate CaMKII, it does so significantly less well than 50 mM KCl, and the majority of this activation is limited to the cell body compartment. In contrast, depolarization caused CaMKII activation in neuronal cell bodies, axons, and dendrites. How then does NGF enhance dendritic growth? Our data indicate that TrkA signaling enhances the KCl-mediated activation of CaMKII and the ERKs (Figure 7E), and we suggest that increased activation of these two pathways causes a further increase in the amount of MAP2 associated with microtubules (as we document here), thereby enhancing dendritic growth. This effect may not be specific to dendritic growth; we have previously shown that NGF-mediated activation of the MEK-ERK pathway is also a key regulator of local axon growth (Atwal et al., 2000).

Dendrite formation is not the only developmental process that is regulated by interactions between neuronal activity and NGF. Our previous work has shown that NGF and KCl can collaborate to promote sympathetic neuron survival via coactivation of a PI3-kinase-Akt survival pathway (Vaillant et al., 1999), consistent with the fact that both afferent input and target contact are essential for maximal sympathetic neuron survival during naturally occurring neuronal death (Maderdrut et al., 1988; Levi-Montalcini, 1987; Kaplan and Miller, 2000). These previous findings, together with the work presented here, indicate that activity and neurotrophins collaborate extensively via intracellular convergence onto key signaling pathways to regulate the survival, maturation, and connectivity of mammalian neurons. We propose that this convergence acts developmentally to allow the selection of only those neurons that reach appropriate targets and establish active circuits, and then acts in the mature nervous system to allow refinement of such connections in response to physiological and environmental inputs.

Experimental Procedures

Neuronal Cell Culture

Sympathetic neurons of the superior cervical ganglion (SCG) were dissected from P1 Sprague-Dawley rats and cultured as described previously (Vaillant et al., 1999). Neurons were cultured onto collagen-coated 96-well plates (2500–3000 cells/well) for survival assays, onto eight-well chamber slides (Nunc) coated first with collagen/poly-D-lysine (Sigma) followed by a second coating of collagen (2500–3000 cells/well) for microscopy, or onto collagen-coated six-well plates (40,000 cells per well) for biochemistry. Neurons were maintained for 5 days in 50 ng/ml NGF (Cedarlane Labs), washed in NGF-free media, and then were switched into 10 ng/ml NGF, 50 mM KCl, 10 ng/ml NGF plus 50 mM KCl, or 10 ng/ml NGF plus 100 μM carbachol. KN-62, mAIP (myristoylated AIP), PD98059, H-89, and forskolin were all obtained from Calbiochem, Inc., while nifedipine was obtained from Biomol. The following concentrations of each of these agents was used in all of the reported experiments: 10 μM KN-62, 100 μM mAIP, 50 μM PD98059, 100 nM H-89, and 10 μM forskolin. PD98059 was replaced every 24 hr in all experiments. MTT assays were performed in 96-well dishes, essentially as previously described (Vaillant et al., 1999). For the field stimulation experiments, neurons were cultured in 24-well plates modified such that four wells were fitted with a pair of platinum electrodes connected in parallel to a stimulator (MultiStim System; Digitimer, Hertfordshire, UK). After 5 days in culture, cells were washed in NGF-free media and then switched to 10 ng/ml NGF and stimulated or not with 0.2 ms constant current (60 mA) pulses of alternating polarity delivered at 5 Hz continuously for 72 hr. A similar stimulation paradigm, applied for 6 hr, has previously been shown to cause alterations in sensory neuron gene expression (Brosenitsch and Katz, 2001).

Immunofluorescence Microscopy

Neurons were cultured in eight-well chamber slides as described above. After treatment, cells were briefly rinsed in phosphate-buffered saline (PBS) (pH 7.2) and fixed for 15 min in 4% paraformaldehyde (BDH) in PBS (pH 7.2). Cells were then permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min and washed three times with PBS. Cells were incubated 1 hr with either the rat monoclonal α-tubulin antibody YOL 1/34 (Serotec), the mouse monoclonal HMW-MAP2 antibody AP20 (Sigma) or the rabbit phospho-CaMKII polyclonal antibody that is directed against the autophosphorylation site at Thr^{286} (Promega). This was followed by a 3×5 min PBS wash

and incubation in either donkey anti-rat DTAF diluted 1:200 in PBS, goat anti-mouse CY3 diluted 1:400 in PBS, or goat anti-rabbit FITC diluted 1:150 in PBS (all from Jackson Laboratories) as required. Cells were then washed three times with PBS and mounted. Fluorescent labeling was visualized on an Axioskop (Zeiss, Inc.) Inverted microscope equipped with a Sony CCD camera. Digital image acquisition and analysis was performed with the Northern Eclipse software (Empix, Inc.).

Morphometry

For each treatment, five HMW-MAP2-labeled fields at 10× magnification were acquired. Each of these fields was processed to normalize the minimum HMW-MAP2 signal. Using NIH image, the lengths of all MAP2-labeled processes were determined by tracing HMW-MAP2-labeled processes from the perimeter of the cell body to the furthest extent of labeling. These numbers were then subjected to histogram analysis using bin sizes of 2 μM. For all treatments, the number of counted processes varied from 200 to 400. The absolute binning values were normalized to percentage of the total population counted to facilitate comparison between treatments. Individual measurements were also used to calculate the mean MAP2-positive process length ± standard error of the mean. Statistics were performed using Student's t test to make pairwise comparisons.

Western Blot Analysis and Quantitative Immunodot Blotting

Sympathetic neurons were rinsed briefly in cold TBS and then lysed as previously described (Vaillant et al., 1999). Protein determinations, Western blot analysis, and detection were all performed as described in Vaillant et al. (1999). Antibodies were used for HMW-MAP2 (mAb AP20, 1:1000), ERK1 and 2 (pAb C-16, Santa Cruz, 1:2500), Ser/Thr-phosphorylated ERK1 and 2 (Promega, 1:2500), Akt and serine-phosphorylated-Akt (both New England Biolabs, 1:1000), and phosphorylated CaMKII (Promega, 1:1000). Secondary antibodies used were HRP-conjugated anti-mouse (1:10000) and anti-rabbit (1:10000) polyclonal antibodies (Boehringer Mannheim). All incubations were performed in 2.5% skim milk in TBS, 0.1% Tween-20 (Sigma).

To quantify the relative amount of HMW-MAP2 associated per μg of tubulin polymer, quantitative determination of tubulin levels and qualitative determination of MAP2 levels were determined in sympathetic neuron extracts essentially as described (Vaillant et al., 1998). As a quantitative tubulin standard, phosphocellulose-purified tubulin was prepared from 3× cycled tubulin extract as described (Weingarten et al., 1975; Lee et al., 1978). As a qualitative MAP2 standard, 3× cycled tubulin extract containing tubulin and microtubule-associated proteins was prepared as described (Weingarten et al., 1975; Collins and Vallee, 1987).

For quantitation of MAP2 phosphorylation, neurons were cultured for 5 days in NGF, washed in NGF-free medium, and then switched to 10 ng/ml NGF with or without 50 mM KCl for 1 min. Cells were lysed in lysis buffer, homogenized by sonication, and protein concentration was determined. 500 μg of protein from each sample was then incubated overnight with 10 μl of a polyclonal antibody for MAP2 (Chemicon) followed by 1 hr incubation with protein A-Sepharose. After centrifugation and washing with lysis buffer, the samples were separated on a 6% polyacrylamide gel, transferred to nitrocellulose, and MAP2 phosphorylation was detected by probing with an antibody that recognizes phosphoserine/threonine (Upstate Biotechnology). To normalize for the total amount of MAP2, 50 μg of protein extract from each sample was separated on a 6% gel, transferred, and probed with anti-MAP2.

Determination of Microtubule Stability

Sympathetic neurons in six-well dishes were washed briefly with 80 mM PIPES (Sigma) (pH 6.8), 5 mM EGTA (Sigma), 1 mM MgCl2 (Sigma) at 37°C. Cells were then incubated for 5 min at 37°C in 250–500 μl of soluble tubulin extraction buffer (0.1M MES [Boehringer] [pH 6.75], 1 mM MgSO4, 2 mM EDTA, 0.1 mM EGTA, 4M glycerol [BDH], 0.5% Triton X-100) (Joshi and Cleveland, 1989) with the addition of Complete Mini Protease Inhibitors (Boehringer). The extraction buffer was then pipetted from the dishes and spun for 2 min at 10,000 rpm at room temperature, and the supernatant was removed and stored at −80°C. Then 250–500 μl of microtubule depolymerizing buffer (0.1M MES [pH 6.9], 1 mM MgSO4, 10 mM CaCl2, 1 mM DTT and 5 mM GTP [type IIs, Sigma]) (Thrower et al., 1991) with the addition of Complete Mini Protease Inhibitors was added to the dish, and the cells were scraped into the Eppendorf tube containing the pellet from the soluble fraction. The cells were then sonicated 2 × 10 s at 94W using a Braun sonicator, incubated 1 hr on ice, and spun 10 min at 10,000 rpm and at 4°C. The supernatant was removed and stored at −80°C. Following protein determination, absolute tubulin levels were quantified using quantitative immunodot blotting as described above. These values were normalized to total protein levels in the soluble and polymerized fractions to yield the percentage of soluble and polymerized tubulin present within the cell.

CaMKII Kinase Activity Assays

Sympathetic neuron lysates (150 μg) were diluted into immunoprecipitation buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% v/v NP-40, 0.5% w/w sodium deoxycholate, 0.1% w/v SDS, 5% v/v glycerol, 10 mM sodium fluoride, 5 mM EGTA, 1 mM EDTA, and 30 mM glycerolphosphate) and incubated with gentle agitation for 4 hr at 4°C in the presence of either antibodies against the α or β subunit of CaMKII. 30 μl of protein A-sepharose beads (Pharmacia) preincubated in cold immunoprecipitation buffer were then added and the sample incubated for an additional 3 hr at 4°C. Beads were centrifuged, washed two times in 3% NETF buffer (100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl [pH 7.4], 50 mM NaF, 1% NP-40) followed by two washes in NETF buffer without NP-40, and a single wash in reaction buffer (20 mM MOPS [pH 7.2], 25 mM β-glycerolphosphate, 5 mM EGTA, 2 mM EDTA, 20 mM $MgCl_2$, 2 mM sodium orthovanadate, 1 mM dithiothreitol, PKA inhibitor peptide [UBI], and 5% glycerol). Beads were then resuspended in 20 μl of reaction buffer and 10 μl of AIP cocktail (2 mg/ml AIP in assay buffer). Reactions were initiated with 10 μl of 250 μM [γ-^{32}P]ATP (~3000 cpm/pmol) in a final volume of 40 μl and incubated for 20 min at 30°C. The reactions were terminated by spotting them on 2.5 cm P81 filter paper (Whatman). The P81 filter papers were washed ten times in 0.75% phosphoric acid and counted for incorporation of radioactivity.

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