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Activity-Dependent Dendritic Arborization Mediated by CaM-Kinase I Activation and Enhanced CREB-Dependent Transcription of Wnt-2

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Summary

Members of the Wnt signaling family are important mediators of numerous developmental events, including activity-dependent dendrite development, but the pathways regulating expression and secretion of Wnt in response to neuronal activity are poorly defined. Here, we identify an NMDA receptor-mediated, Ca^{2+}-dependent signaling pathway that couples neuronal activity to dendritic arborization through enhanced Wnt synthesis and secretion. Activity-dependent dendritic outgrowth and branching in cultured hippocampal neurons and slices is mediated through activation by CaM-dependent protein kinase kinase (CaMKK) of the membrane-associated γ isoform of CaMKI. Downstream effectors of CaMKI include the MAP-kinase pathway of Ras/MEK/ERK and the transcription factor CREB. A serial analysis of chromatin occupancy screen identified Wnt-2 as an activity-dependent CREB-responsive gene. Neuronal activity enhances CREB-dependent transcription of Wnt-2, and expression of Wnt-2 stimulates dendritic arborization. This novel signaling pathway contributes to dynamic remodeling of the dendritic architecture in response to neuronal activity during development.

Introduction

Proper dendritic arborization is a critical process for accurate development of neuronal circuits and for activity-dependent plasticity in mature neurons (Libersat and Duch, 2004; Scott and Luo, 2001; Yuste and Bonhoeffer, 2001). Indeed, dendritic abnormalities are the most consistent pathologic correlate of mental retardation including Down's syndrome (Becker et al., 1986; Benavides-Piccione et al., 2004) and Fragile X syndrome (O'Donnell and Warren, 2002). Extension, retraction, and branching of dendritic processes, as well as stabilization of these processes by synaptogenesis, are extremely complex functions that include intrinsic genetic programs (Jan and Jan, 2003) and influences from extrinsic factors (McAllister et al., 1999).

One extrinsic factor recently demonstrated to stimulate dendritic development is Wnt (Rosso et al., 2005; Yu and Malenka, 2003). Wnt is a secreted glycoprotein that binds to the Frizzled family of receptors to activate the scaffold protein Dishevelled. This event in turn activates signaling pathways essential for multiple aspects of neuronal development (Ciani and Salinas, 2005; Nusse and Varmus, 1992). The pathways downstream of the Wnt/Dishevelled cascade that regulate dendrite development include GSK3β-mediated stabilization of β-catenin (Yu and Malenka, 2003) and stimulation of the Rac pathway (Rosso et al., 2005). Elevated β-catenin (Gumbiner, 1996) and activated Rac (Van Aelst and Cline, 2004) are known to modulate the actin cytoskeleton, an important event in dendritic outgrowth (Miller and Kaplan, 2003). Although the signaling pathways downstream of Wnt that regulate dendrite arborization are being defined, little is known about pathways that regulate Wnt synthesis and secretion. This lack of knowledge has been an obstacle to defining the signaling processes governing dendritic arborization.

This study identifies a Ca^{2+}-dependent pathway activated by neuronal activity that is essential for dendritic outgrowth and branching through stimulating the transcription of Wnt-2. Neuronal activity is critical for maturation of mammalian dendrites (Konur and Ghosh, 2005; Wong and Ghosh, 2002). Maximal dendrite growth and remodeling occurs during afferent innervation (Wu et al., 1996), and dendrite development is impaired under conditions of afferent deprivation (McAllister et al., 1996). In addition, dendrite development requires Ca^{2+} signaling through calmodulin-dependent protein kinases (CaMKs) (Cline, 2001; Konur and Ghosh, 2005) For example, it is known that CaMKII activity is inversely related to the growth rate of dendrites in the optic tectum of the *Xenopus* tadpole (Wu and Cline, 1998; Zou and Cline, 1999). In addition to CaMKII, neurons contain the CaMK cascade, consisting of CaMK kinase (CaMKK) and its principal downstream targets CaMKI and CaMKIV (Means, 2000; Soderling, 1999). CaMKIV is predominantly nuclear (Jensen et al., 1991), where it regulates gene transcription through phosphorylation of CREB and CBP (Enslen et al., 1994; Impey et al., 2002). In contrast, CaMKI is nuclear excluded (Picciotto et al., 1995) and distributes throughout the cell soma, including the axon and dendrites of neurons (Wayman et al., 2004). Several in vitro substrates of CaMKI have been identified; these include synapsin, (Nairn and Greengard, 1987), myosin II regulatory light chair, (Suizu et al., 2002), translation initiation factor 4GII (Qin et al., 2003), and numb family proteins, (Tokumitsu et al., 2005). Recent studies have identified roles for CaMKI in mediating axonal outgrowth and growth cone motility (Wayman et al., 2004) and in the induction of early-phase long-term potentiation (E-LTP; Schmitt et al., 2005) through its ability to activate the MAP-kinase ERK (Schmitt et al., 2004). This crosstalk between CaMKI and the MAP-kinases necessitates examination of other physiological roles that may be subserved by this pathway.

Numerous studies using KCl depolarization to mimic neuronal activity imply obligatory roles for both a CaMK and ERK in activity-dependent dendritic development using KCl depolarization to mimic neuronal activity. For example, depolarization of sympathetic neurons

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in the presence of NGF stimulates dendritic outgrowth that is dependent on the activity of both CaMKII and ERK (Vaillant et al., 2002). In depolarized cultured hippocampal neurons, βCaMKII, but not αCaMKII, activity increases filopodial motility and branching at DIV 3 but is inhibitory at DIV 11 (Fink et al., 2003). This specificity for βCaMKII versus αCaMKII is due to its ability to interact with the actin cytoskeleton. Moreover, repetitive depolarizations of hippocampal dentate gyrus stimulate the formation of dendritic filopodia in a NMDAR-dependent manner that requires activation of both a CaMK and ERK (Wu et al., 2001b). Activation of ERK and CaMK is also required for promoting neurite growth under conditions of prolonged depolarization in cultured cortical neurons (Redmond et al., 2002). In that study, expression of constitutively active (ca) CaMKIV phenocopies the effect of depolarization whereas expression of caCaMKII is inhibitory. The MEK inhibitor U0126 also inhibits neurite outgrowth, indicating involvement of ERK. Neurite outgrowth may also require CREB-dependent gene transcription since dominant-negative (dn) CREB blocks the effect of depolarization. Together, the evidence suggests dendrites require CaMK and ERK for proper development. However, the identification of which CaMKs may be involved, their relationship to ERK and CREB, and the gene target(s) regulated by CREB are not clear. These ambiguities are clarified in this study. We found that activity-dependent dendritic arborization required sequential activation of the NMDAR, CaMKK, CaMKI, and MEK/ERK to enhance CREB-mediated transcription of Wnt-2.

Results

The CaMK Cascade Selectively Regulates Activity-Dependent Dendritic Development

As noted above, several reports have identified an obligatory dual role for a CaMK and ERK in mammalian dendrite development. We specifically investigated the possible involvement of CaMKK and CaMKI in dendritic arborization for two reasons. First, we have shown that Ca^{2+}-dependent activation of ERK via the NMDAR in hippocampal neurons requires the CaMKK/CaMKI pathway (Schmitt et al., 2004), providing a likely explanation for the dual requirement for ERK and a CaMK in dendrite development. Second, the CaMKK/CaMKI pathway plays a critical role in the outgrowth of the axon (Wayman et al., 2004). We initially studied cultured hippocampal neurons between days 7 and 9 because this time period is critical for dendritic elongation and branching (see Figure S1 in the Supplemental Data available with thist article online).

Because neuronal activity has a major effect on dendritic arborization (Nimchinsky et al., 2002; Volkmar and Greenough, 1972; Wong and Ghosh, 2002), we incubated cultured neurons with 16 mM KCl between days 7 and 9 to induce neuronal activity (Yu and Malenka, 2003). Dendrite development was selectively measured by expression of an EGFP-Map2B construct on DIV 7 under the control of the *CAG* promoter that shows neuron-specific expression (S. Kaech, personal communication). Furthermore, high molecular weight Map2 is strongly expressed in dendrites but not axons (Tucker and Matus, 1988). Dendritic length and branching were then visualized on DIV 9 (Figure S2, top right panel). Expression of EGFP-Map2B was restricted to the dendrites and was not present in the axon, as illustrated by coexpression of the axonal marker Ng-CaM with EGFP-Map2B (Figure S2, lower right panel). The total level of Map2B in the transfected neuron (Figure S2, upper right panel) was not greatly enhanced over neighboring nontransfected neurons (upper left panel), indicating that expression of exogenous Map2B was modest relative to endogenous Map2B. None of the treatment paradigms in this study increased apoptosis as measured by Hoechts staining (data not shown).

Upon addition of 16 mM KCl or 20 μM bicuculline, a GABA-A channel inhibitor that also enhances excitatory neuronal activity, we observed an increase of 70%–100% in both total dendritic length and branching between days 7 and 9 (Figures 1A–1C). These activity-dependent increases in dendritic length and branching were blocked by treatment with the NMDAR antagonists APV (Figure 1D) and 7-Cl-kynuric acid (data not shown) but not by the L-type Ca^{2+} channel inhibitor nifedipine (Figure 1D). These results support the conclusion that enhanced dendrite development in response to neuronal activity is mediated by the Ca^{2+}-permeable NMDAR.

To probe for potential involvement of Ca^{2+}-dependent CaMKK in activity-enhanced dendritic development, we tested whether the CaMKK inhibitor STO-609 suppressed the stimulatory effect of 16 mM KCl. We have previously shown that treatment of cultured hippocampal neurons with 2–5 μM STO-609 inhibits CaMKK but not CaMKII (Wayman et al., 2004). As shown in Figures 1E and 1F, STO-609 selectively blocked the stimulatory effect of KCl with no effect on basal outgrowth or branching. The fact that STO-609 treatment had no effect on basal dendrite development (see Figure S1) or the motility of immature dendritic growth cones (Figure S3 and Movies S1–S3) is important since STO-609 strongly suppresses basal axonal outgrowth and growth cone motility (Wayman et al., 2004). These observations verify that STO-609 does not have nonspecific effects (e.g., on the cytoskeleton) during development of neuronal processes.

Although STO-609 appears to be highly selective for inhibition of CaMKK in vitro (Tokumitsu et al., 2002) and in cultured neurons (Wayman et al., 2004), it could be exerting its effect on activity-dependent dendrite development by inhibiting some unknown protein other than CaMKK. To address this possibility, a CaMKK mutant (L233F) that is largely insensitive to STO-609 (Tokumitsu et al., 2003) was expressed to determine if, as previously observed (Schmitt et al., 2005; Wayman et al., 2004), it would rescue inhibition by STO-609. Expression of this CaMKK mutant had no effect on basal or KCl-stimulated dendritic arborization, but it did reverse the inhibition by STO-609 (Figure 1F). These data provide strong evidence that STO-609 suppressed dendritic development through its inhibition of CaMKK.

Having established a critical role for CaMKK, we next investigated its major downstream effectors, CaMKI and CaMKIV, using dominant-negative constructs previously characterized (Schmitt et al., 2005; Wayman et al., 2004). The dnCaMKI, but not dnCaMKIV, suppressed activity-dependent enhancement of dendritic growth and branching with no effect on basal parameters (Figures

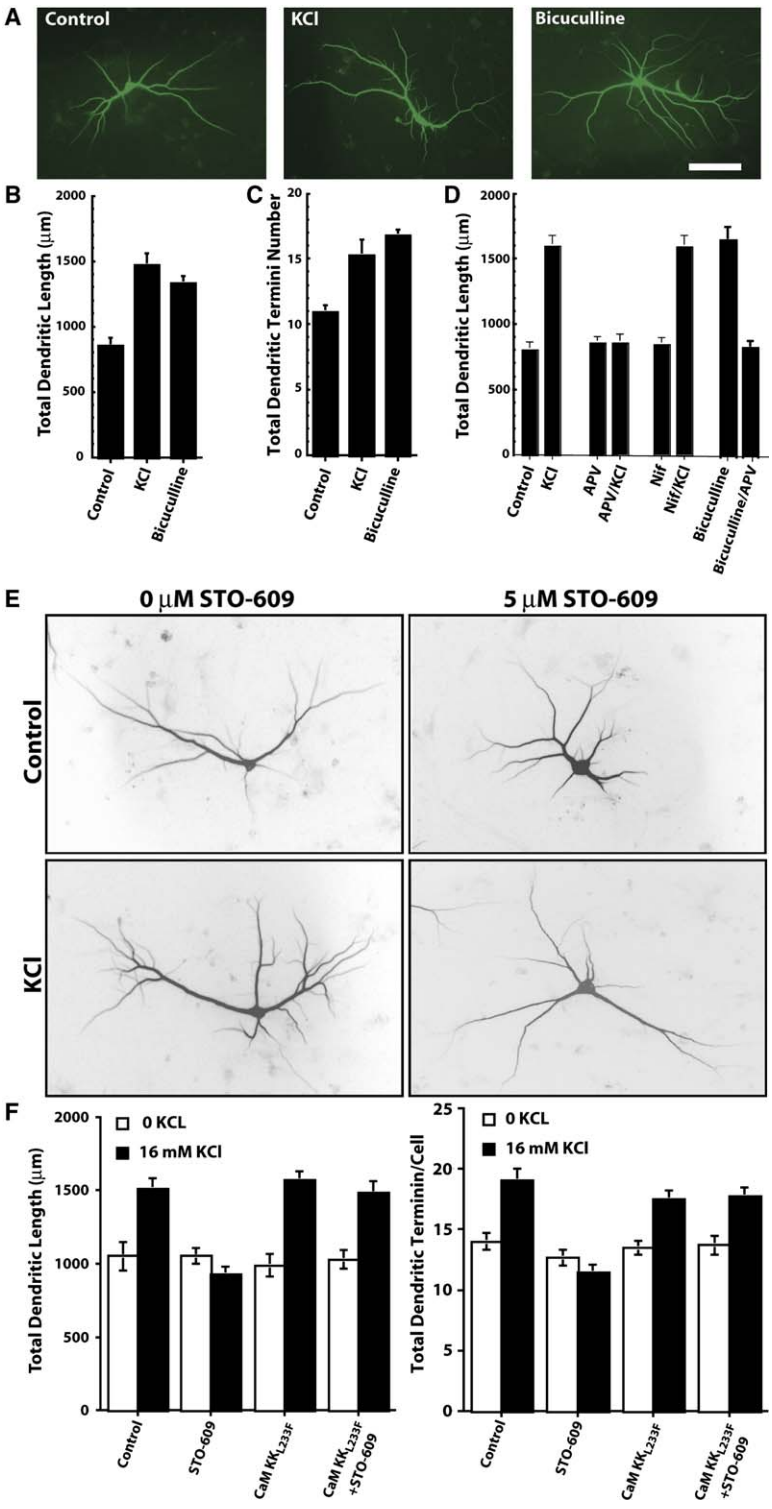

Figure 1. Activity-Dependent Dendritic Growth Requires the NMDA Receptor and CaMKK

(A) Representative images of 9 DIV hippocampal neurons expressing EGFP-Map2B following 48 hr treatment with either vehicle alone (control), 16 mM KCl, or 20 μM bicuculline (Scale bar: 100 μm). Effects of these treatments on dendritic length (B) and branching (C) were quantified using EGPF-Map2B expression (Figure S2) to identify dendrites (30 cells per condition, mean ± SEM).

(D) Inhibition of the NMDAR blocked activity-dependent outgrowth. EGFP-Map2B expressing hippocampal neurons were treated with vehicle, 16 mM KCl, 20 μM bicuculline alone or in combination with either 50 μM D-APV (NMDAR antagonist) or 10 μM nifedipine (Nif, L-type calcium channel blocker) for 48 hr. Average total dendritic length (± SEM) is shown.

(E) Hippocampal neurons (7 DIV) were transfected with EGFP-Map2B, treated with 16 mM KCl ± 5 μM STO-609 for 48 hr, and fixed. Representative examples of control and treated neurons are shown.

(F) Neurons were transfected with EGFP-Map2B without or with cotransfected STO-609 insensitive CaMKK (L233F) and treated as in (E). Average total dendritic length (left) and branching (right) (±SEM) were quantified. Note that the inhibition by STO-609 was rescued by the STO-609-insensitive mutant of CaMKK.

2A and 2B). We used our dnCaMKIVnuc containing a nuclear localization signal (NLS) to restrict its expression to the nucleus (Wayman et al., 2004), thereby mimicking endogenous CaMKIV (Jensen et al., 1991). The dnCaMKIV construct without the NLS is improperly expressed throughout the neuron (Wayman et al., 2004) and therefore may elicit nonphysiological responses, probably by suppressing CaMKK which has several downstream effectors. Expression of a constitutively active CaMKI (caCaMKI) phenocopied the effect of activity-dependent stimulation (Figure 2C). The stimulatory effect of caCaMKI was not blocked by the NMDAR antagonist APV (data not shown), consistent with CaMKI being downstream of the NMDAR.

Lastly, we investigated which CaMKI isoforms may be involved in dendrite development by expressing siRNA

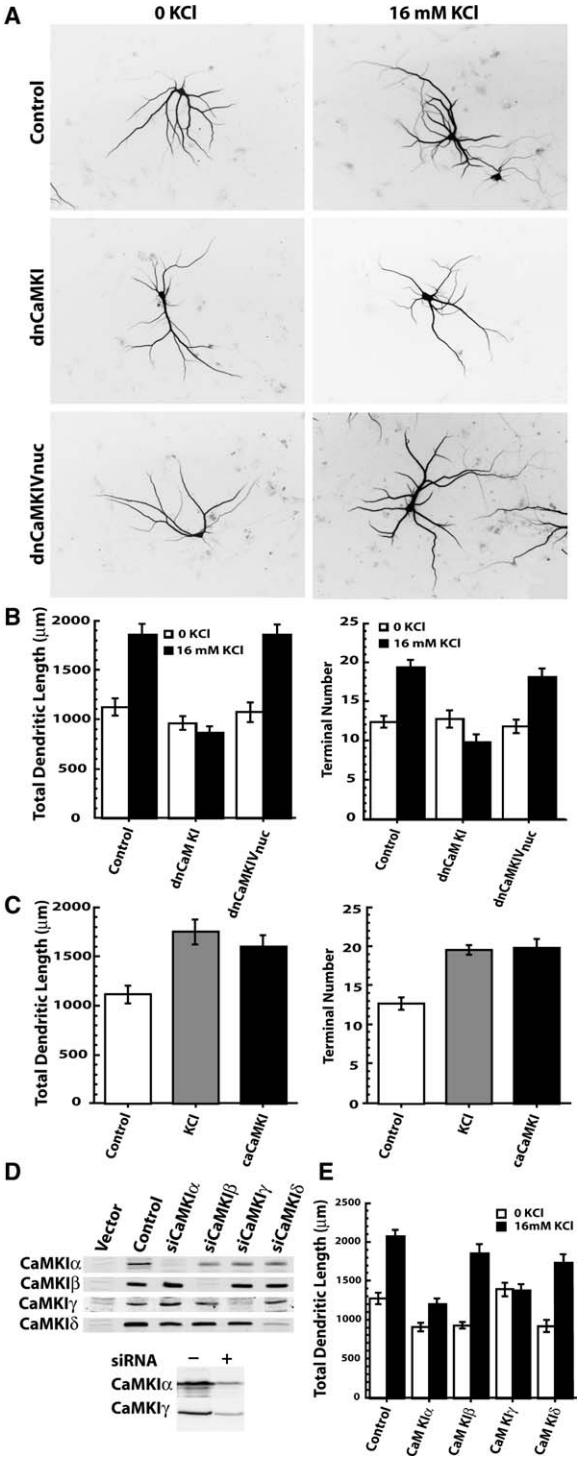

Figure 2. Activity-Dependent Dendritic Growth Requires CaMKI but Not CaMKIV

Hippocampal neurons (7 DIV) were transfected with plasmids encoding EGFP-Map2B alone (control) or in combination with dominant-negative (dn) or constitutively active (ca) CaMKI or CaMKIVnuc (containing a NLS to restrict expression to the nucleus), cultured an additional 48 hr ± 16 mM KCl, and then fixed and imaged.

(A) Representative examples of control and dnCaMK-expressing neurons ± 16 mM KCl.

(B) Quantification of the effects of dnCaMKs on activity-dependent dendrite development.

(C) Constitutively active CaMKI alone was sufficient to stimulate dendritic growth and branching. Hippocampal neurons (7 DIV) were transfected with EGFP-Map2B alone or in combination with caCaMKI. Following transfection, control neurons were treated ± 16 mM KCl for 48 hr. Average dendritic length and branching (± SEM) were determined as before (30 cells per condition).

(D) Top: siRNAs block expression of CaMKI isoforms. HEK293 cells were cotransfected with FLAG-tagged CaMKI isoforms α, β, δ, or γ ± siRNAs for their respective isoforms. After 24 hr, cell lysates were analyzed by Western blots to determine expression levels of the CaMKs. Note that each siRNA specifically suppresses expression of only its isoform. Bottom: Hippocampal neurons were transfected with FLAG-tagged α or γ CaMKI ± their respective siRNAs and lysates were analyzed by Western blot.

(E) siRNAs for α and γ CaMKI block dendrite development. Hippocampal neurons (5 DIV) were transfected with the indicted siRNA (control is a scrambled siRNA) plus EGFP-Map2B and then stimulated with 16 mM KCl on 7 DIV to assess dendritic development at 9 DIV.

constructs that suppress the individual CaMKI isoforms, all of which are present in brain. Experiments in HEK293 cells demonstrated the specificity of the siRNAs. Each siRNA effectively blocked expression of its respective CaMKI isoform without effecting expression of the other isoforms (Figure 2D, top). The siRNAs against both α and γ isoforms blocked dendritic development (Figure 2E), as well as overexpressed FLAG-tagged α and γ CaMKI, respectively (Figure 2D, bottom), whereas the siRNAs for β and δ had no effect (Figure 2E). Taken together, results from these multiple techniques all strongly suggest that CaMKK is acting through the α and γ isoforms of CaMKI to enhance activity-dependent dendritic development.

The MAP-Kinase Cascade Is Downstream of CaMKK/CaMKI

We have previously demonstrated crosstalk between CaMKK/CaMKI and the MAP-kinase MEK/ERK pathway. KCl depolarization of NG108 neuroblastoma cells activates ERK and stimulates neurite outgrowth, both of which are mediated by CaMKK/CaMKI via Ras (Schmitt et al., 2004). NMDAR activation of ERK in hippocampal neurons also involves CaMKK/CaMKI as does the ERK-dependent component of E-LTP (Schmitt et al., 2005). These findings, in combination with numerous studies indicating a requirement for ERK in neuronal development (see Introduction), suggest that the Ras/MEK/ERK pathway may be essential for CaMKK/CaMKI-mediated activity-dependent dendritic maturation.

To investigate this possibility, we treated neurons with 20 μM bicuculline or 16 mM KCl, and both protocols resulted in a 2-fold activation of ERK, as determined using a phosphospecific antibody against its activation site by MEK. ERK activation occurred within 10 min and was sustained for greater than 90 min (Figure S4) and was blocked by the MEK inhibitor U0126 and by the NMDA antagonist D-APV (Figure 3A). Since our earlier experiments showed that the siRNAs for α and γ CaMKI suppressed dendritic outgrowth, they were tested for their ability to block activity-dependent ERK activation. The siRNA for γ, but surprisingly not the α isoform, was effective (Figure 3B).

Having established activation of ERK by CaMKI under our activity-dependent stimulation paradigms, we next investigated the role of MEK/ERK in dendritic outgrowth. Indeed, U0126 suppressed KCl-mediated outgrowth (Figure 3C), and constitutively active forms of

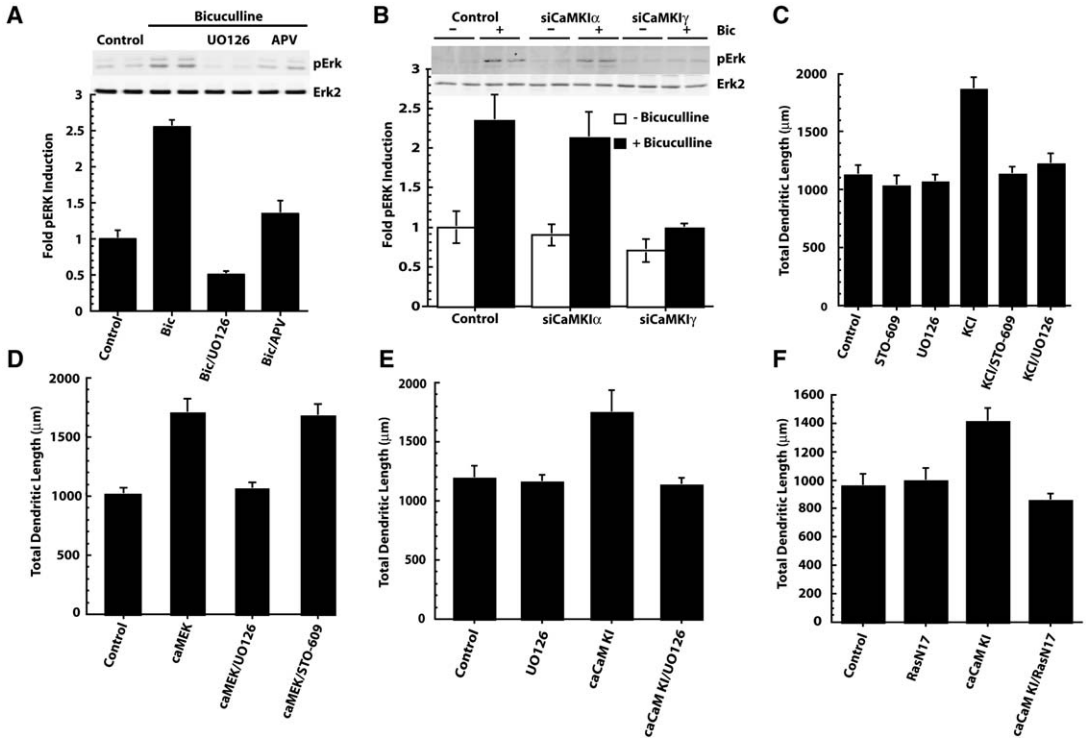

Figure 3. Requirements for CaMKK/CaMKI and Activation the MAP-Kinase Pathway

(A) Activation of ERK by bicuculline. Hippocampal neurons (7 DIV) were stimulated with 20 μM bicuculline for 5 min in the absence or presence of either 10 μM U0126 or 50 μM D-APV. Endogenous ERK activation was assessed by the level of phospho-ERK (pErk) normalized to total ERK (Erk2).

(B) siRNA for γ CaMKI blocked ERK activation. Neurons (5 DIV) were transfected with siRNA for α or γ CaMKI (control is scrambled siRNA) plus a vector expressing FLAG-ERK, stimulated on DIV 7 for 5 min with bicuculline, and immunoprecipitated FLAG-ERK activation was determined using the phosphospecific antibody as in (A).

(C) Inhibition of MEK blocked KCl-stimulated dendritic outgrowth. Hippocampal neurons (7 DIV) expressing EGFP-Map2B were treated ± 16 mM KCl, ± 5 μM STO-609, or ± 10 μM U0126. After 48 hr of drug treatment, average total dendritic length was determined (30 cells per condition).

(D) Activation of MEK is sufficient to stimulate dendritic outgrowth. Hippocampal neurons (7 DIV) were cotransfected with EGFP-Map2B and control vector or constitutively active MEK (caMEK) and treated with U0126 or STO-609 as indicated. At DIV 9, average total dendritic length was determined.

(E) CaMKI-mediated dendritic growth requires MEK. Hippocampal neurons were cotransfected with EGFP-Map2B and control vector or caCaMKI ± U0126 treatment for 2 days.

(F) Expression of dominant interfering Ras (RasN17) blocked CaMKI-mediated dendritic growth. Hippocampal neurons (7 DIV) were cotransfected with EGFP-Map2B, ± RasN17, ± caCaMKI as indicated. On DIV 9, dendritic growth was quantified (±SEM, 30 cells per condition).

MEK (Figure 3D) or CaMKI (Figure 3E) phenocopied the effects of KCl or bicuculline in stimulating dendrite development. Lastly, the dominant-interfering mutant of Ras (RasN17) also blocked CaMKI-mediated outgrowth (Figure 3F). These results are consistent with a signaling pathway involving the NMDAR, CaMKK/CaMKI, and Ras/MEK/ERK.

Role of CREB-Mediated Transcription

Many Ca^{2+}-dependent effects mediated by MAP-kinases involve gene transcription through phosphorylation of the transcription factor CREB (Impey et al., 1999; Impey et al., 1998; Thomas and Huganir, 2004). It has been reported that dnCREB blocks KCl-stimulated dendritic outgrowth in cortical neurons (Redmond et al., 2002). Furthermore, NMDA-mediated synaptic activity elicited by bicuculline treatment rapidly phosphorylates CREB, an event that is blocked by inhibitors of CaMKs and MEK (Lee et al., 2005). Therefore, we investigated the role of CREB in activity-dependent dendritic arborization.

As expected, treatment of neurons with either KCl or bicuculline resulted in rapid (<10 min) and sustained (>90 min) phosphorylation of the activation site (Ser133) in CREB (data not shown) which was blocked by U0126 (Figure 4A). Dendrite outgrowth stimulated by KCl, bicuculline, caCaMKI, and caMEK were all suppressed by a dominant-interfering CREB (ACREB; Figure 4B). Importantly, expression of a siRNA that strongly suppressed CREB expression in neurons (Figure 4C) had little effect on basal dendritic development, but it completely blocked arborization induced by neuronal activity (Figure 4D).

We investigated the role of specific CaMKI isoforms in CREB-dependent transcription using siRNAs since this approach demonstrated involvement of the α and γ but not the β or δ isoforms in dendritic outgrowth (Figure 2E). CREB-dependent transcription, measured using a CRE-luciferase reporter gene, was specifically blocked by siRNA for γ but not α CaMKI (Figure 4E), identical to their effects on ERK activation (Figure 3B). Basal transcription was not affected by the siRNA

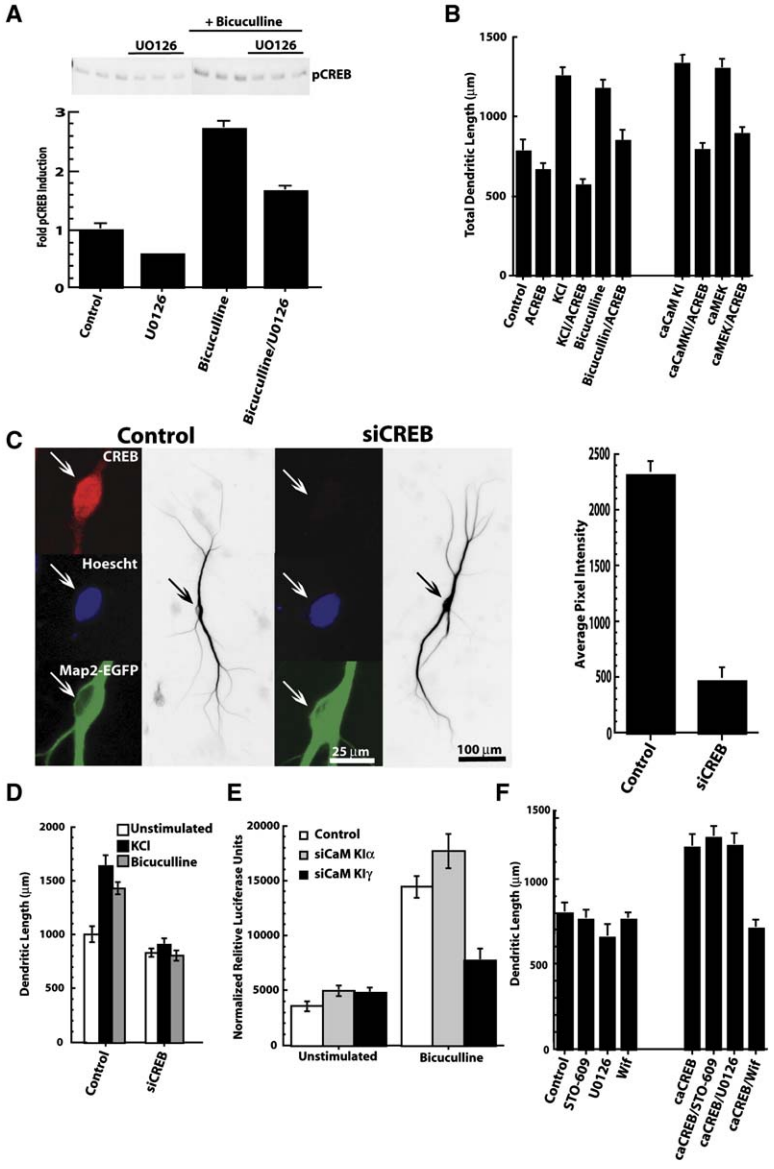

Figure 4. Role of CREB-Dependent Transcription in Dendritic Outgrowth

(A) Activity-dependent CREB phosphorylation requires MEK. Hippocampal neurons (7 DIV) were stimulated for 30 min with 20 μM bicuculline ± U0126, and CREB phosphorylation (S133) was quantified.

(B) Inhibition of CREB-mediated transcription blocked activity-dependent dendritic development. Hippocampal neurons (7 DIV) were cotransfected with EGFP-Map2B and control vector or either dominant-interfering CREB (ACREB), caCaMKI, or caMEK. Control cells were then treated ±16 mM KCl. At DIV 9, average total dendritic length was determined (±SEM, 30 cells per condition).

(C) Endogenous CREB expression was strongly suppressed by siRNA for CREB. Hippocampal neurons (5 DIV) were cotransfected with EGFP-Map2B and either control vector or siRNA for CREB. On DIV 7, the neurons were fixed and stained for endogenous CREB (red) or Hoescht staining for DNA (purple). Representative neurons are shown. The bars on the right quantify the levels of endogenous CREB (n = 30).

(D) Suppression of endogenous CREB blocks activity-dependent dendrite development. Hippocampal neurons (5 DIV) were transfected with EGFP-Map2B ± siRNA for CREB and on DIV 7 were treated with ±16 mM KCl or ±20 μM bicuculline, and on DIV 9 dendritic arborization was quantified (±SEM, 30 cells per condition).

(E) siRNA of γCaMKI blocks CREB-mediated transcription. Neurons (5 DIV) were transfected with control (scrambled) or siRNAs for α or γCaMKI plus a CRE-luciferase reported gene construct. On DIV 7, they were stimulated for 4 hr with 20 μM bicuculline and lysed, then luciferase levels were quantified (n = 4).

(F) Activation of CREB is sufficient to stimulate dendritic growth via Wnt. Hippocampal neurons (7 DIV) were transfected with EGFP-Map2B, ± caCREB (CREB-DIEDML) or ± the secreted Wnt inhibitor Wif and treated with 5 μM STO-609 or 20 μM U0126. At DIV 9, average total dendritic length was determined (±SEM, 30 cells per condition).

for γ CaMKI (Figure 4E); nor were BDNF-stimulated CREB-dependent or NFκB-dependent transcription (data not shown). Transfection with a caCREB (CREB-DIEDML) phenocopied the effects of neuronal activity on dendritic outgrowth, and this stimulatory effect was not suppressed by STO-609 or U0126 (Figure 4F). These results indicated that CREB functions downstream of CaMKK and MEK.

Identification of Wnt-2 as a CREB-Responsive Gene

Because there are approximately 19 members of the Wnt family, we utilized a nonbiased serial analysis of chromatin occupancy (SACO) screen for CREB target regions to identify candidate CREB-regulated genes that have been implicated in dendritic outgrowth. Although other candidate genes were regulated by activators of CREB, Wnt-2 showed a high degree of responsiveness to neuronal activity. The Wnt-2 SACO locus was associated with a promoter-proximal CpG island and conserved CREB response element (CRE1), both of which suggest a role in transcriptional regulation (Figures 5A and 5B). The Wnt-2 SACO locus was quantitatively immunoprecipitated from hippocampal neuron chromatin by CREB antisera (Figure 5C). A control region in the GAPDH promoter was not detected whereas Mkp1, another CREB-responsive gene (Impey et al., 2004), was also immunoprecipitated, indicating the specificity of this approach. Stimulation of hippocampal neurons with forskolin and KCl triggered a rapid rise in Wnt-2 message (Figure 5D). The ability of these CREB activators to stimulate Wnt-2 transcription was markedly attenuated by expression of ACREB, a potent inhibitor of CREB function. Furthermore, the increase in Wnt-2 message induced by bicuculline treatment was blocked by U0126 and STO-609 (Figure 5E). These results indicate that the Wnt-2 promoter is occupied by CREB in vivo and that Wnt-2 transcription is inducibly regulated by the CREB pathway downstream of CaMKK and ERK.

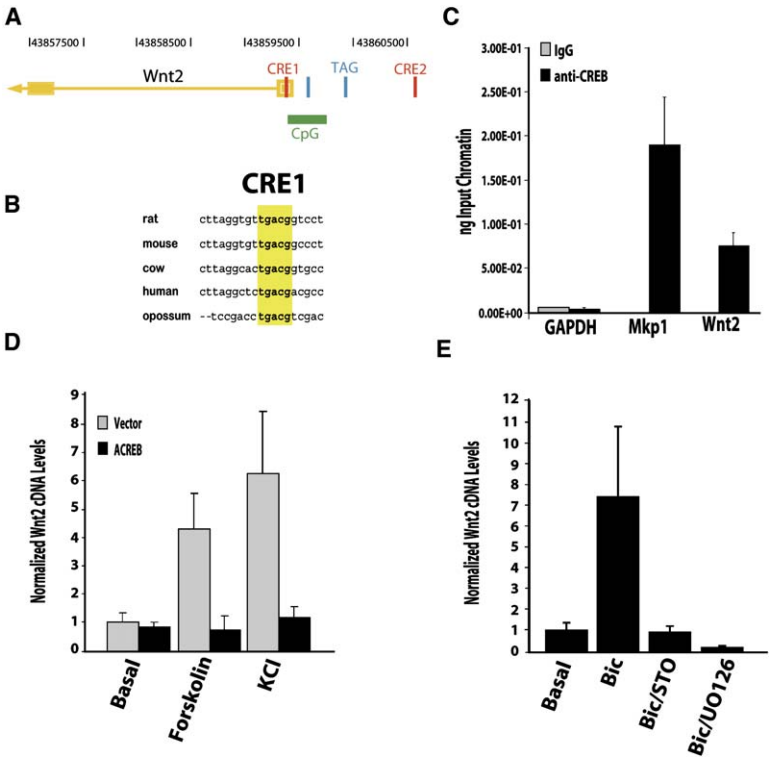

Figure 5. Wnt-2 Is a CREB-Responsive Gene

(A) A diagram indicating the relative positions of SACO tags (blue), CRE (TGACG) motifs (red), CpG islands (green), and the Wnt-2 gene along Chr 4 (UCSC Rn3 assembly).

(B) Phylogenetic conservation of the CRE2 site in the first exon of Wnt-2.

(C) Rat hippocampal neurons were subjected to chromatin immunoprecipitation (ChIP assay) using a CREB antibody or IgG control. Real-time PCR was conducted using primers that interrogate the Wnt-2 SACO locus, the Mkp1 promoter, and a control region in the GAPDH gene (±SEM, n = 4).

(D) Induction of Wnt-2 requires CREB. Hippocampal neurons were transfected with dominant-negative CREB (ACREB) or vector control and treated 36 hr later with forskolin or KCl for 60 min. RNA was reverse transcribed and analyzed by real-time PCR with Wnt-2 cDNA primers. The data were normalized to GAPDH cDNA levels (±SEM, n = 6).

(E) Wnt expression requires CaMKK and MEK. Hippocampal neurons (7 DIV) were stimulated with 20 μM bicuculline for 2 hr ± 10 μM U0126 or 5 μM STO-609.

A previous report shows that dendrite development stimulated by 16 mM KCl is blocked by a Wnt antagonist, but the species of Wnt was not identified (Yu and Malenka, 2003). To ascertain whether our CaMKI signaling pathway is upstream of Wnt, we used the Wnt inhibitor Wif, a secreted protein that binds Wnt and prevents it from activating its receptor Frizzled (Han and Lin, 2005). Expression of Wif completely suppressed dendritic arborization induced by KCl, bicuculline, caCaMKI, caMEK (Figure 6A) as well as by caCREB (Figure 4F) without effects on basal outgrowth. These results show that the CaMKK/CaMKI, MEK/ERK, and CREB-responsive components of our signaling pathway are upstream of Wnt.

Having established that Wnt-2 is a CREB-responsive gene, we tested whether it could directly stimulate dendrite development. Neurons treated with conditioned media from Wnt-2 expressing HEK293 cells (data not shown) or transfected with a construct that expressed Wnt-2 exhibited enhanced dendritic length and branching, similar to that observed with neuronal activity, and the Wnt-2-dependent outgrowth of the transfected neurons was blocked by coexpression of Wif (Figure 6B).

Identification of Wnt-2 as a CREB-responsive gene whose transcription is enhanced by neuronal activity suggests it may contribute to programs of gene expression that regulate morphogenesis and structural plasticity. To further explore the role of Wnt-2 in neuronal development, we measured its temporal expression in rat hippocampus. Wnt-2 mRNA levels from whole hippocampus were very low at P1 but exhibited a dramatic increase at P7–P14 and then declined to slightly elevated levels in the adult (Figure 7A). This result was verified and expanded by in situ hybridization which demonstrated that hippocampal slices from E18 and P1 pups showed little Wnt-2 mRNA, strong expression occurred at P7 and P14 in regions CA1, CA2, and CA3 followed by a sharp declined in the adult except for region CA1 (Figure 7B). Thus, Wnt-2 expression was induced during the period of active afferent input and dendrite development in intact hippocampus (Pokorny and Yamamoto, 1981; Wong and Ghosh, 2002).

Dendritic Development in Organotypic Slices

To extend our results obtained in cultured neurons to a more physiological system, we investigated key steps in activity-dependent dendritic arborization using cultured hippocampal slices. Organotypic slices retain most of the cellular and morphological organization of the intact hippocampus, except for the absence of afferent input, and have been used extensively to study numerous aspects of hippocampal function including plasticity and development (Bahr, 1995; Caeser and Aertsen, 1991). Hippocampal slices from P5 rat pups were cultured for 3 days, subjected to biolistic transfection, allowed to recover for 1 day, and then treated with 16 mM KCl or 20 μM bicuculline for 2 days to mimic afferent input followed by fixation (see Experimental Procedures). Transfected neurons were visualized with tomato fluorescent protein (TFP) because of its strong expression (Figure 8A), and dendritic length of CA1 pyramidal neurons, which exhibit strong Wnt-2 expression (Figure 7B), was analyzed using confocal microscopy. Treatment with either 16 mM KCl or 20 μM bicuculline produced a 50%–70% increase in dendritic length and branching (data not shown) that were blocked by prior treatment with either STO-609 or U0126 (Figure 8B). Expression of siRNAs for α or γCaMKI or for CREB, as well as expression of dominant-negative CREB, all suppressed bicuculline-stimulated outgrowth (Figure 8C). Treatment with bicuculline for 4 hr induced strong

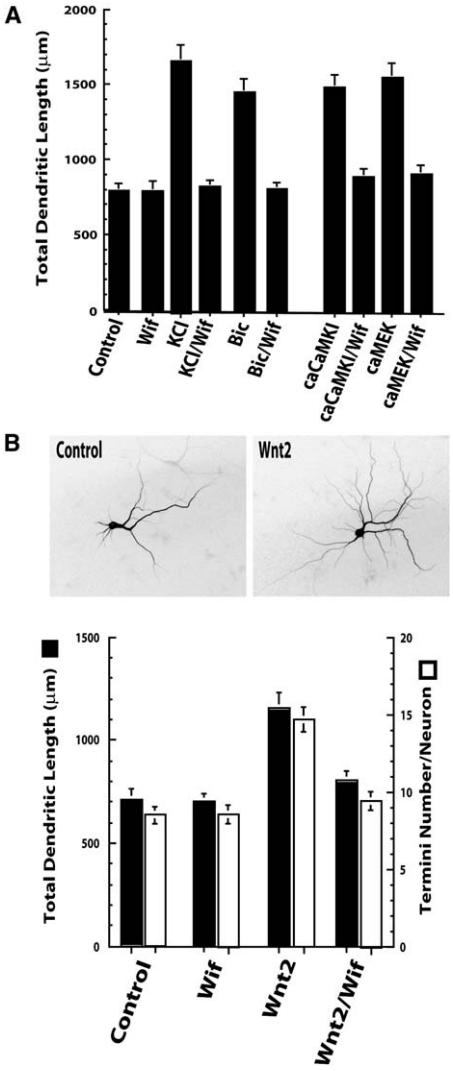

Figure 6. Activity-Dependent Dendritic Growth Is Mediated by Wnt

(A) Activity-, CaMKI-, and MEK-stimulated dendritic growth was mediated by Wnt. Hippocampal neurons (7 DIV) were cotransfected with EGFP-Map2B ± Wif, caCaMKI, or caMEK. Control cells were treated with either ±16 mM KCl or 20 μM bicuculline. At DIV 9 total dendritic length was determined (±SEM, 30 cells per condition).

(B) Wnt-2 stimulated dendritic arborization. Hippocampal neurons (7 DIV) were cotransfected with EGFP-Map2B alone or in combination with either Wnt-2 or Wif. At DIV 9 average dendritic length and branching were determined as shown in the bottom panel (±SEM, 30 cells per condition). The top panel illustrates representative neurons.

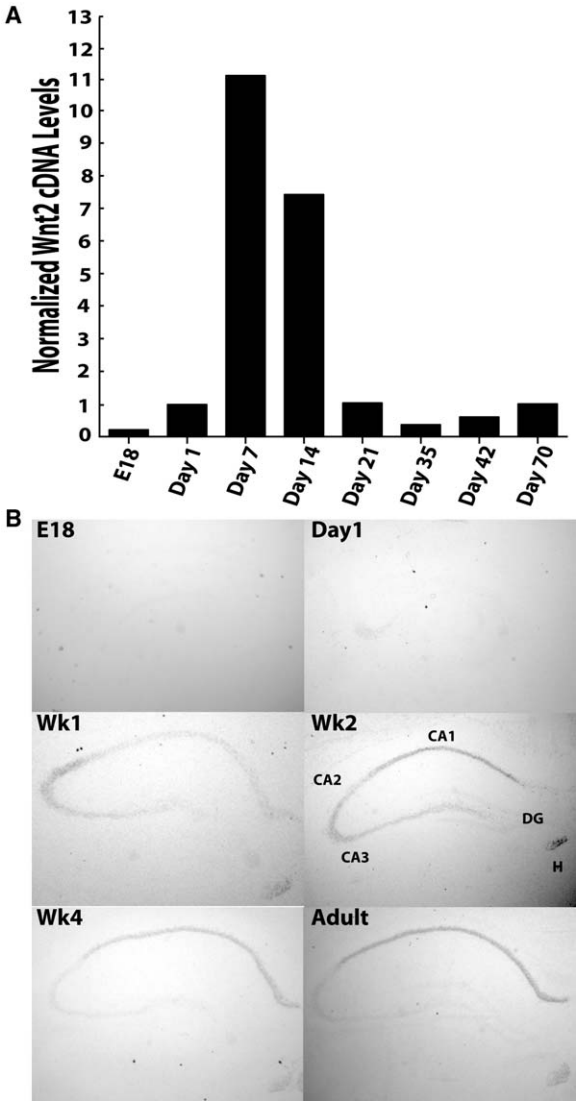

Figure 7. Expression of Wnt-2 in Rat Hippocampus

(A) Wnt-2 mRNA is enhanced within the hippocampus during afferent innervation. Hippocampi were isolated from rats (two animals/developmental time point, E18-adult). RNA was isolated, reverse transcribed, and analyzed by real-time PCR with Wnt-2 cDNA primers. The data are normalized to GAPDH cDNA levels.

(B) Wnt-2 is strongly expressed within hippocampal regions CA1, CA2, and CA3. Developmental expression pattern of Wnt-2 mRNA was determined by in situ hybridization of coronal sections at the level of the dorsal hippocampus (E18-adult). Representative photomicrographs are shown (CA1, CA2, CA3, Dentate Gyrus [DG], Habenula [H]).

transcription of Wnt-2, and this was obviated by treatment with either U0126 or STO-609 (Figure 8D). Lastly, expression of the Wnt antagonist Wif blocked bicuculline-dependent outgrowth whereas expression of Wnt-2 itself was sufficient to enhance dendritic length (Figure 8E). Thus, we conclude that activity-dependent dendrite development in cultured hippocampal slices, as in cultured neurons, requires the CaMKK/CaMKI/ERK/CREB/Wnt-2 pathway.

Discussion

Proper development of the complex network of dendrites is essential to their function as integrators of multiple synaptic inputs. In recent years, several studies have identified neuronal activity (Wong and Ghosh, 2002), mediated largely by Ca^{2+} signaling (Konur and Ghosh, 2005), as one of several key modulators of dendritic arborization, and these investigations also implicated involvement of a member of the CaMK family as well as the MAP-kinase ERK (Fink et al., 2003; Redmond et al., 2002; Vaillant et al., 2002; Wu et al., 2001b). However, the relevant CaMK was not definitively identified because these studies used a single pharmacological inhibitor or molecular approach, each of which by itself has serious caveats. For example, KN-62 and KN-93

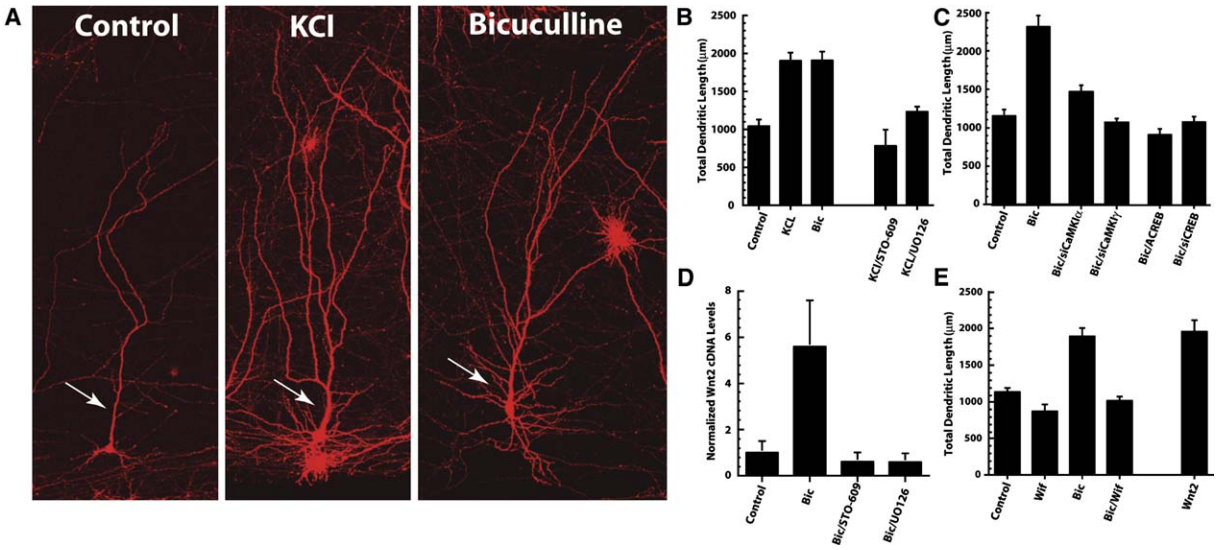

Figure 8. Regulation of Dendritic Development in Cultured Hippocampal Slices

Hippocampal slices from P5 rats were cultured (see Experimental Procedures) for 3 days, subjected to biolistic transfection with TFP ± other plasmids as indicated, allowed to recover for 1 day, and then treated for 2 days with either ±16 mM KCl or 20 μM bicuculline and other pharmacological agents as indicated. Total dendritic length of CA1 pyramidal neurons was determined (±SEM, 20 cells per condition).

(A) Representative examples (see arrows) of control or stimulated CA1 pyramidal neurons are shown.

(B) Activity-stimulated dendritic growth in organotypic slices required CaMKK and MEK. TFP-transfected slices were treated as indicated ±20 μM bicuculline or ±16 mM KCl ± 5 μM STO-609, ±10 μM U0126 for 2 days.

(C) Requirements for CaMKIα, CaMKIγ, and CREB. Slices were cotransfected with TFP ± siCaMKIα, ± siCaMKIγ ± ACREB, or ± siCREB and stimulated ± 20 μM bicuculline.

(D) Activity increased the expression of Wnt-2. Organotypic slices cultured for 3 days were treated with either 5 μM STO-609 or 10 μM U0126 for 4 hr and then stimulated with 20 μM bicuculline for 4 hr. RNA was reverse transcribed and analyzed by real-time PCR with Wnt-2 cDNA primers. The data were normalized to GAPDH cDNA levels (±SEM, n = 5–6).

(E) Wnt-2 stimulates dendritic arborization. Organotypic slices were transfected with TFP ± Wif ± Wnt-2 and then cultured for 2 days with 20 μM bicuculline.

are not specific CaMKII inhibitors (Enslen et al., 1994; Mochizuki et al., 1993) as previously claimed (Tokumitsu et al., 1990), and expressed dnCaMKIV, in the absence of an added NLS, is mislocalized in the cytosol where it can exert nonphysiological effects (Wayman et al., 2004). Furthermore, the link between CaMK and ERK was also not known. In light of our recent studies showing that CaMKK and CaMKI can activate ERK (Schmitt et al., 2004), we investigated the role of this pathway in dendrite development. Lastly, the downstream target of the CaMK/ERK pathway had not been identified. Here, we have demonstrated, based on multiple independent experimental approaches including use of pharmacological inhibitors, dominant-interfering and constitutively active signaling proteins, and siRNAs, a crucial role for CaMKK and CaMKI in mediating activity-dependent dendritic development in cultured hippocampal neurons between 7 and 9 DIV. Our study has delineated a signaling pathway triggered by neuronal activity that sequentially activates NMDARs, CaMKK, CaMKI, Ras, MEK/ERK, and CREB-dependent transcription of Wnt-2. Identification of this signaling pathway upstream of Wnt is important, not only because Wnt is a known effector of dendritic arborization (Rosso et al., 2005; Yu and Malenka, 2003), but in a broader context Wnt has pleiotropic effects on developmental patterning, stem cell renewal, and diseases such as cancer (Logan and Nusse, 2004). This CaMKK/CaMKI, Ras/ERK, and CREB pathway may be involved in regulating these Wnt-dependent phenomena also.

It is interesting that the siRNAs for both α and γCaMKI suppressed dendrite development (Figure 2E) whereas only the γ isoform was involved in ERK activation (Figure 3B) and CREB-dependent transcription (Figure 4E). Involvement of γCaMKI makes sense because this isoform contains a C-terminal CAAX motif and is associated with the plasma membrane, probably through a prenylation-dependent mechanism (Takemoto-Kimura et al., 2003). Ras also contains a CAAX motif, and its membrane localization is required for proper activation (Hancock, 2003). However, the mechanism by which αCaMKI regulates dendrite development remains to be elucidated. One possibility is that it could phosphorylate the myosin II regulatory light chain (Suizu et al., 2002) to regulate the dendritic cytoskeleton. Alternatively, the Numb family, which regulate axonal outgrowth (Nishimura et al., 2003) and dendritic spine development (Nishimura et al., 2006) and are also substrates of CaMKI (Tokumitsu et al., 2005), may be an effector of αCaMKI.

It was somewhat surprising that CREB-dependent dendritic development in response to neuronal activity was not blocked by nuclear-localized dnCaMKIV (Figure 2B). This construct is known to attenuate CREB-dependent transcription in response to stronger stimuli (e.g., 50 mM KCl; Wayman et al., 2004). CaMKIV is nuclear localized (Jensen et al., 1991), is an excellent CREB kinase (Enslen et al., 1995), and also stimulates transcription through direct phosphorylation of the CREB binding protein CBP (Impey et al., 2002). Furthermore, mice lacking CaMKIV show deficits in CREB

phosphorylation in response to strong stimuli (e.g., 50 mM KCl, 100 μM glutamate) or behavioral tests (Ho et al., 2000; Ribar et al., 2000). In contrast to these CaMKIV findings, our activity-dependent protocols (16 mM KCl or 20 μM bicuculline) stimulated CREB-dependent transcription through CaMKI, a known activator of the MAP kinases MEK/ERK. MSK and possibly RSK, which are downstream of ERK, catalyze prolonged phosphorylation of CREB that is essential for transcription (Arthur et al., 2004; Impey et al., 1998; Wu et al., 2001a). Thus, different CaMKs appear to participate in phosphorylation of CREB, depending on the stimulation paradigm.

It appears that several members of the CaMK family play important functions at different temporal phases of dendritic development in various types of neurons. Extensive studies in *Xenopus* optic tectum (Wu and Cline, 1998; Zou and Cline, 1999) have established that activating CaMKII reduces the rate of dendritic outgrowth through stabilizing the dendritic arbor late in dendritic development. In depolarized (50 mM KCl) hippocampal neurons, βCaMKII has a stimulatory role on filopodial motility early in development (3 DIV) but, as in the *Xenopus* optic tectum, is inhibitory at later times (11 DIV) when synaptogenesis is prominent (Fink et al., 2003). In another study, repetitive 3 min depolarizations (90 mM KCl) produced prolonged ERK activation that stimulated filopodial formation on dendrites (Wu et al., 2001b). The general CaMK inhibitor KN-93 blocked both ERK activation and filopodial stimulation, again indicating involvement of both pathways. We propose these effects are mediated by CaMKK/CaMKI.

Likewise, depolarization (50 mM KCl) of cortical neurons (2–4 DIV) promotes dendritic growth that requires activation of both ERK and a CaMK acting through CREB-mediated transcription (Redmond et al., 2002). The authors concluded this kinase is CaMKIV because expression of caCaMKIV phenocopied the effect of depolarization on dendritic outgrowth. The different conclusions between their work and ours may relate to several variables: (1) the use of cortical versus hippocampal neurons; (2) the developmental age of cultures (3–4 DIV versus 7–9 DIV); (3) stimulation paradigms (50 mM KCl versus 16 mM KCl or 20 μM bicuculline); and (4) expression of CaMKIV constructs without or with a NLS to restrict CaMKIV to the nucleus. For example, their use of 50 mM KCl evoked activation of L-type Ca^{2+} channels, whereas our stimulation paradigms utilized NMDARs. This difference may be important, since Ca^{2+}-dependent signaling pathways utilized by voltage-gated Ca^{2+} channels and NMDARs can be different (Bading et al., 1993).

A previous study using cultured hippocampal neurons concluded that activity-dependent (e.g., KCl depolarization) dendrite development is mediated largely through Wnt signaling, and they focused on the signaling pathway downstream of Wnt involving β-catenin (Yu and Malenka, 2003). Since the stimulatory effects of all our reagents were blocked by the Wnt antagonist Wif (Figures 4F and 6A), we concur on the essential role of Wnt and identified Wnt-2 as a major mediator, but we focused on the previously unknown upstream signaling events coupling the NMDAR and Ca^{2+} influx to Wnt synthesis. Because CREB-dependent transcription was upstream of Wnt (Figure 4F), we employed chromatin immunoprecipitation assays and found that the proximal promoter of the Wnt-2 gene contained multiple CREB-responsive elements. Depolarization-stimulated Wnt-2 transcription was also attenuated by cotransfection of dnCREB, indicating that Wnt-2 is a bona fide CREB-responsive gene (Figure 5D). Thus, Wnt-2 represents one of the first CREB-regulated rapid response genes that is capable of directly regulating morphological plasticity. For example, neuronal differentiation of NT2 cells by retinoic acid, which stimulates CREB phosphorylation via ERK (Canon et al., 2004), dramatically elevates expression of Wnt-2 (Katoh, 2002). Our study expands previous results on dendritic development in cultured neurons by confirming this signaling pathway in cultured hippocampal slices which retain most of the morphological organization of the intact hippocampus (Bahr, 1995; Caeser and Aertsen, 1991). In situ hybridization of hippocampus demonstrated a dramatic increase in Wnt-2 in regions CA1–CA3 in P7–P14 rat pups that corresponds to the period of active afferent input and dendrite development in intact hippocampus (Pokorny and Yamamoto, 1981; Wong and Ghosh, 2002). Since our study utilized cultured hippocampal slices which are devoid of most afferent input, we used KCl or bicuculline to mimic neuronal activity. Our results are consistent with the hypothesis that the elevated Wnt-2 in hippocampus between P7 and P14 may result from enhanced afferent activity and is important in proper dendritic arborization. Acute increases in electrical activity in the hippocampus promotes neurogenesis, enhances Wnt-2 expression, and elevates levels of β-catenin (Madsen et al., 2003).

While our data clearly demonstrate involvement of Wnt-2 in mediating activity-dependent dendrite development, we cannot rule out possible roles of other Wnt isoforms. Wnt binding to the family of Frizzled receptors, which recruits the scaffold protein Dishevelled (Dvl), can trigger multiple signaling pathways (Ciani and Salinas, 2005). Several of these pathways regulate the actin cytoskeleton, which is probably involved in modulating dendritic morphology during development. In the canonical Wnt pathway, Dvl activation blocks GSK3β, decreasing the phosphorylation of β-catenin (Salic et al., 2000).

Because degradation of β-catenin in proteosomes by the ubiquination pathway is enhanced by its phosphorylation, inhibiting GSK3β enhances the levels of β-catenin. Such elevation of β-catenin can have two major consequences. First, it can associate with nuclear transcription factors to stimulate gene transcription, but this transcription pathway downstream of Wnt/Dvl does not appear to be involved in dendritic outgrowth (Yu and Malenka, 2003). Second, β-catenin can also bind the intracellular domain of N-cadherin, as well as associate with α-catenin, an actin binding protein, to stabilize the dendritic cytoskeleton (Bamji, 2005). This scenario has been implicated in activity-dependent dendritic outgrowth (Yu and Malenka, 2003). For example, expressing a fragment of N-cadherin that binds β-catenin in an abortive complex blocks activity-dependent dendritic outgrowth (Yu and Malenka, 2003).

Wnt signaling has also been investigated in early (DIV 2–5) hippocampal cultures under basal conditions (Rosso et al., 2005). In this case, binding of Wnt-7b to Frizzled signals through the scaffold protein Dvl to activate the RhoGTPase, Rac. Rac activates JNK to

stimulate dendritic arborization, perhaps through regulation of both actin and stabilization of microtubules (Chang et al., 2003). Thus, basal levels of Wnt may regulate dendritic development through a Rac/JNK pathway. Furthermore, in the Rosso et al. (2005) study, the Wnt antagonist Sfrp1 suppressed by 45% basal dendritic development, whereas in our experiments, Wif selectively inhibited activity-dependent outgrowth. This difference may reflect differences in stages of neuronal development or culture conditions. Our results demonstrate that neuronal activity further enhances dendritic arborization through an NMDAR-dependent signaling pathway consisting of CaMKK, CaMKI, Ras, ERK, and CREB-dependent transcription of Wnt-2. It will be of interest to determine if this same pathway is upstream of Wnt signaling in its effects on other developmental patterning.

Experimental Procedures

Cell Culture

High-density hippocampal neurons (2×10^5 cells per square centimeter) were cultured according to the procedure of Brewer (Brewer, 1997) from P0–2 Sprague Dawley rats on plates coated with poly-L-lysine (Sigma; molecular weight 300,000). The hippocampal neurons were maintained in Neurobasal A media (Invitrogen) supplemented with B27 (Invitrogen) and 0.5 mM L-glutamine with 5 μM cytosine-D-arabinofuranoside (AraC) added at 2 DIV. The hippocampal neurons were then cultured a further 3–5 days, at which time they were either transfected or treated with various pharmacological reagents as specified in the text or figure legends.

Transfection and Reporter Assays

Primary hippocampal neurons were transfected with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocols. In each experiment, we optimized DNA amounts, transfection reagent amounts, and transfection duration to minimize toxicity and maximize transfection efficiency. None of the transfections or drug treatments described in this work had noticeable effects on apoptosis as assessed by condensed nuclei using Hoechst staining. Luciferase and β-galactosidase activities were measured using Luciferase and Galacoto-Light assay kits (Perkin Elmer).

Microscopy and Quantification of Dendrite Outgrowth

After transfection, cells were allowed to grow for varying times (6 hr to 2 days), fixed with 4% paraformaldehyde, 4% sucrose, 50 mM HEPES, PBS (pH 7.5), for 10–15 min at room temperature, and then permeabilized with 0.25% Triton X-100 in PBS. Cells were viewed on a Zeiss Axiophot or a Leica DM RXA microscope with high numerical aperture lenses, and the images were recorded with a cooled CCD camera. Total process lengths were measured using Openlabs software.

Slice Culture and Transfection

Organotypic hippocampal slices from P5 Sprague-Dawley rats were cultured for 3 days as described (Barria and Malinow, 2002). To visualize dendritic arbors, slices were transfected with pCAG-TFP (± test plasmids) using a Helios Gene Gun (BioRad), according to the manufacturer's protocol. Following transfection, slices were allowed to recover for 24 hr before stimulation with 16 mM KCl or 20 μm bicuculline for 2 days. Slices were fixed, mounted, and imaged using a confocal microscope. Dendritic processes were measured as described above.

In Situ Hybridization

E18-adult Sprague-Dawley rats used for in situ hybridization were anesthetized and then decapitated. Following decapitation, brains were isolated and frozen on powdered dry ice. Coronal sections (20 μm) were then thaw mounted onto SuperFrost Plus slides (VWR Scientific) and stored at −80ºC.

Slides with rat brain sections were processed before hybridization as follows (all steps at room temperature): 5 min fix in 4% paraformaldehyde in 0.1 M phosphate buffer, acetylated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated through a graded series of ethanol, delipidated for 5 min in chloroform, rehydrated in a second ethanol series, and air dried. Hybridization buffer containing radiolabeled probes were applied to the tissue. Slides were then covered with glass coverslips, placed in a humid chamber and incubated overnight at 55ºC. The following day, slides were treated with RNase A and washed under conditions of increasing stringency, including two 30 min washes in 0.1× SSC (sodium saline citrate; 15 mM NaCl, 1.5 mM sodium citrate) at 60ºC. Slides were then dehydrated through ethanol containing 300 mM ammonium acetate and air dried. Slides were dipped in Kodak NTB-2 liquid emulsion diluted 1:1 with distilled water, allowed to air dry for 1 hr, and then stored in the dark at 4ºC. After sufficient exposure time, the slides were developed and coverslipped.

To generate an antisense riboprobe for Wnt-2, a fragment of the Wnt-2 gene (nt 352–652) was generated by PCR from rat Wnt2 cDNA. The Wnt2 PCR product was subcloned into *pGEM-EZ-T*. Radiolabeled antisense probe was generated with a T7-based in vitro transcription kit. Sense probe was generated with a SP6 based in vitro transcription kit.

Antibodies and Plasmids

The following reagents were purchased from the indicated sources: U0126, Calbiochem; STO-609, NMDA, and APV from Tocris Cookson. Antibodies were purchased from the following sources: phospho-ERK1/2 (Thr202, Tyr204), Cell Signaling Technology; phospho-CREB (Ser133), gift of Michael Greenberg; ERK2 (D-2) from Santa Cruz Biotechnology; and Flag (M2) from Sigma. Construction of Map2B-GFP and Wnt-2 constructs was as follows: Map2B and Wnt-2 cDNAs were isolated using standard PCR base method from total rat brain cDNA. Map2B-GFP was constructed by fusing EGFP to the C-terminal of Map2B and then subcloning the fusion construct into pCAGGS expression vector. Wnt-2 was subcloned into *pCS2*$^+$. dnCaMKI (K49E, T177A, lHQS286DDEE, F307A), dnCaMKIV nuclear (T196A, K71E, HMDT305DEDD), and caCaMKI (HMDT305DEDD) plasmids have been described previously (Wayman et al. 2004). The FLAG-ERK2, RasN17 plasmids were provided by Dr. Philip Stork (Vollum Institute, Portland, Oregon), *pCAG*-ACREB, *pCAG*-LacZ, and CRE-Luciferase were described previously (Arthur et al., 2004).

siRNA Constructs

All siRNA constructs were cloned into *p702P* vector: siCaMKIα, *CTT GTG TAA GAC GGC GAT CTC*; siCaMKIβ, *CCA AGT GGA CTG ACT CCT A*; siCaMKIγ, *CCG AGC ACT TCC ATG AAG ATG*; siCaMKIδ, *ATG GAT CGC TGG TGA CAC A*; siCREB, *TTC TGC AAT AGT TGA AAT CTG*.

Chromatin Immunoprecipitation

$1–2 \times 10^6$ neonatal day 7 rat hippocampal neurons were subjected to chromatin immunoprecipitation as previously described (Impey et al., 2004).

Reverse Transcription

Neurons were treated as described, and total RNA was isolated using Trizol (Invitrogen) according to manufacturer's instructions. RNA (50 ng to 3 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) and 50–250 ng random primers (Invitrogen).

Quantitative PCR

Primers were designed using MIT's Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with default parameters except the following: rodent and simple repeat library was on, product size was 50–200 bp, primer size was 18–27 bases, Tm was 66ºC–72ºC, maximum self-complementarity was 5, and maximum 3′ complementarity was 3. For analysis of cDNA levels near a tag, primers were selected within the nearest downstream UTR or exon that had extensive mRNA or spliced EST evidence. Primer sequences are available on request. PCRs (10 μl) contained 1 μl 10× PCR buffer (Invitrogen), 2.5 mM MgCl2, 200 μM dNTP (Roche),

0.125–0.25 μM primer (IDT), 1× SYBR green I (Invitrogen), and 1 U platinum *Taq* (Invitrogen). PCR was run on an Opticon OP346 (MJ Research) for one cycle at 95ºC, 35 s, and 30–50 cycles at 94ºC, 15 s; 68ºC–70ºC, 40 s. CREB ChIPs were expressed as either ng of purified input DNA. RT-PCR experiments were normalized to 18S RNA levels (other housekeeping genes showed similar results). All RT-PCR generated 100-fold higher levels of product than no reverse transcriptase controls.

Supplemental Data

Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/50/6/897/DC1/.

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References

Arthur, J.S., Fong, A.L., Dwyer, J.M., Davare, M., Reese, E., Obrietan, K., and Impey, S. (2004). Mitogen- and stress-activated protein kinase 1 mediates cAMP response element-binding protein phosphorylation and activation by neurotrophins. J. Neurosci. *24*, 4324–4332.

Bading, H., Ginty, D.D., and Greenberg, M.E. (1993). Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science *260*, 181–186.

Bahr, B.A. (1995). Long-term hippocampal slices: a model system for investigating synaptic mechanisms and pathologic processes. J. Neurosci. Res. *42*, 294–305.

Bamji, S.X. (2005). Cadherins: Actin with the cytoskeleton to form synapses. Neuron *47*, 175–178.

Barria, A., and Malinow, R. (2002). Subunit-specific NMDA receptor trafficking to synapses. Neuron *35*, 345–353.

Becker, L.E., Armstrong, D.L., and Chan, F. (1986). Dendritic atrophy in children with Down's syndrome. Ann. Neurol. *20*, 520–526.

Benavides-Piccione, R., Ballesteros-Yanez, I., de Lagran, M.M., Elston, G., Estivill, X., Fillat, C., Defelipe, J., and Dierssen, M. (2004). On dendrites in Down syndrome and DS murine models: a spiny way to learn. Prog. Neurobiol. *74*, 111–126.

Brewer, G.J. (1997). Isolation and culture of adult rat hippocampal neurons. J. Neurosci. Methods *71*, 143–155.

Caeser, M., and Aertsen, A. (1991). Morphological organization of rat hippocampal slice cultures. J. Comp. Neurol. *307*, 87–106.

Canon, E., Cosgaya, J.M., Scsucova, S., and Aranda, A. (2004). Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol. Biol. Cell *15*, 5583–5592.

Chang, L., Jones, Y., Ellisman, M.H., Goldstein, L.S., and Karin, M. (2003). JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtubule-associated proteins. Dev. Cell *4*, 521–533.

Ciani, L., and Salinas, P.C. (2005). WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. Nat. Rev. Neurosci. *6*, 351–362.

Cline, H.T. (2001). Dendritic arbor development and synaptogenesis. Curr. Opin. Neurobiol. *11*, 118–126.

Enslen, H., Sun, P., Brickey, D., Soderling, S.H., Klamo, E., and Soderling, T.R. (1994). Characterization of Ca^{2+}/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. J. Biol. Chem. *269*, 15520–15527.

Enslen, H., Tokumitsu, H., and Soderling, T.R. (1995). Phosphorylation of CREB by CaM-kinase IV activated by CaM-kinase IV kinase. Biochem. Biophys. Res. Commun. *207*, 1038–1043.

Fink, C.C., Bayer, K.U., Myers, J.W., Ferrell, J.E., Jr., Schulman, H., and Meyer, T. (2003). Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. Neuron *39*, 283–297.

Gumbiner, B.M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell *84*, 345–357.

Han, C., and Lin, X. (2005). Shifted from Wnt to Hedgehog signaling pathways. Mol. Cell *17*, 321–322.

Hancock, J.F. (2003). Ras proteins: different signals from different locations. Nat. Rev. Mol. Cell Biol. *4*, 373–384.

Ho, N., Liauw, J.A., Blaeser, F., Wei, F., Hanissian, S., Muglia, L.M., Wozniak, D.F., Nardi, A., Arvin, K.L., Holtzman, D.M., et al. (2000). Impaired synaptic plasticity and cAMP response element-binding protein activation in Ca^{2+}/calmodulin-dependent protein kinase type IV/Gr-deficient mice. J. Neurosci. *20*, 6459–6472.

Impey, S., Obrietan, K., Wong, S.T., Poser, S., Yano, S., Wayman, G., Deloulme, J.C., Chan, G., and Storm, D.R. (1998). Cross talk between ERK and PKA is required for Ca^{2+} stimulation of CREB-dependent transcription and ERK nuclear translocation. Neuron *21*, 869–883.

Impey, S., Obrietan, K., and Storm, D.R. (1999). Making new connections: Role of ERK/MAP kinase signaling in neuronal plasticity. Neuron *23*, 11–14.

Impey, S., Fong, A.L., Wang, Y., Obrietan, K., Wayman, G.A., Storm, D.R., Soderling, T.R., and Goodman, R.H. (2002). Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM kinase IV. Neuron *34*, 235–244.

Impey, S., McCorkle, S.R., Cha-Molstad, H., Dwyer, J.M., Yochum, G.S., Boss, J.M., McWeeney, S., Dunn, J.J., Mandel, G., and Goodman, R.H. (2004). Defining the CREB regulon: A genome-wide analysis of transcription factor regulatory regions. Cell *119*, 1041–1054.

Jan, Y.N., and Jan, L.Y. (2003). The control of dendrite development. Neuron *40*, 229–242.

Jensen, K.F., Ohmstede, C.A., Fisher, R.S., and Sahyoun, N. (1991). Nuclear and axonal localization of Ca^{2+}/calmodulin-dependent protein kinase type Gr in rat cerebellar cortex. Proc. Natl. Acad. Sci. USA *88*, 2850–2853.

Katoh, M. (2002). Regulation of WNT signaling molecules by retinoic acid during neuronal differentiation in NT2 cells: threshold model of WNT action (review). Int. J. Mol. Med. *10*, 683–687.

Konur, S., and Ghosh, A. (2005). Calcium signaling and the control of dendritic development. Neuron *46*, 401–405.

Lee, B., Butcher, G.Q., Hoyt, K.R., Impey, S., and Obrietan, K. (2005). Activity-dependent neuroprotection and cAMP response element-binding protein (CREB): kinase coupling, stimulus intensity, and temporal regulation of CREB phosphorylation at serine 133. J. Neurosci. *25*, 1137–1148.

Libersat, F., and Duch, C. (2004). Mechanisms of dendritic maturation. Mol. Neurobiol. *29*, 303–320.

Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. *20*, 781–810.

Madsen, T.M., Newton, S.S., Eaton, M.E., Russell, D.S., and Duman, R.S. (2003). Chronic electroconvulsive seizure up-regulates beta-catenin expression in rat hippocampus: role in adult neurogenesis. Biol. Psychiatry *54*, 1006–1014.

McAllister, A.K., Katz, L.C., and Lo, D.C. (1996). Neurotrophin regulation of cortical dendritic growth requires activity. Neuron *17*, 1057–1064.

McAllister, A.K., Katz, L.C., and Lo, D.C. (1999). Neurotrophins and synaptic plasticity. Annu. Rev. Neurosci. *22*, 295–318.

Means, A.R. (2000). Regulatory cascades involving calmodulin-dependent protein kinases. Mol. Endocrinol. *14*, 4–13.

Miller, F.D., and Kaplan, D.R. (2003). Signaling mechanisms underlying dendrite formation. Curr. Opin. Neurobiol. *13*, 391–398.

Mochizuki, H., Ito, T., and Hidaka, H. (1993). Purification and characterization of Ca^{2+}/calmodulin-dependent protein kinase V from rat cerebrum. J. Biol. Chem. *268*, 9143–9147.

Nairn, A.C., and Greengard, P. (1987). Purification and characterization of Ca^{2+}/calmodulin-dependent protein kinase I from bovine brain. J. Biol. Chem. *262*, 7273–7281.

Nimchinsky, E.A., Sabatini, B.L., and Svoboda, K. (2002). Structure and function of dendritic spines. Annu. Rev. Physiol. *64*, 313–353.

Nishimura, T., Fukata, Y., Kato, K., Yamaguchi, T., Matsuura, Y., Kamiguchi, H., and Kaibuchi, K. (2003). CRMP-2 regulates polarized Numb-mediated endocytosis for axon growth. Nat. Cell Biol. *5*, 819–826.

Nishimura, T., Yamaguchi, T., Tokunaga, A., Hara, A., Hamaguchi, T., Kato, K., Iwamatsu, A., Okano, H., and Kaibuchi, K. (2006). Role of numb in dendritic spine development with a Cdc42 GEF intersectin and EphB2. Mol. Biol. Cell *17*, 1273–1285.

Nusse, R., and Varmus, H.E. (1992). Wnt genes. Cell *69*, 1073–1087.

O'Donnell, W.T., and Warren, S.T. (2002). A decade of molecular studies of fragile X syndrome. Annu. Rev. Neurosci. *25*, 315–338.

Picciotto, M.R., Zoli, M., Bertuzzi, G., and Nairn, A.C. (1995). Immunochemical localization of calcium/calmodulin-dependent protein kinase I. Synapse *20*, 75–84.

Pokorny, J., and Yamamoto, T. (1981). Postnatal ontogenesis of hippocampal CA1 area in rats. I. Development of dendritic arborisation in pyramidal neurons. Brain Res. Bull. *7*, 113–120.

Qin, H., Raught, B., Sonenberg, N., Goldstein, E.G., and Edelman, A.M. (2003). Phosphorylation screening identifies translational initiation factor 4GII as an intracellular target of Ca2+/calmodulin-dependent protein kinase I. J. Biol. Chem. *278*, 48570–48579.

Redmond, L., Kashani, A.H., and Ghosh, A. (2002). Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. Neuron *34*, 999–1010.

Ribar, T.J., Rodriguiz, R.M., Khiroug, L., Wetsel, W.C., Augustine, G.J., and Means, A.R. (2000). Cerebellar defects in Ca^{2+}/calmodulin kinase IV-deficient mice. J. Neurosci. *20*, RC107.

Rosso, S.B., Sussman, D., Wynshaw-Boris, A., and Salinas, P.C. (2005). Wnt signaling through Dishevelled, Rac and JNK regulates dendritic development. Nat. Neurosci. *8*, 34–42.

Salic, A., Lee, E., Mayer, L., and Kirschner, M.W. (2000). Control of beta-catenin stability: Reconstitution of the cytoplasmic steps of the wnt pathway in *Xenopus* egg extracts. Mol. Cell *5*, 523–532.

Schmitt, J.M., Wayman, G.A., Nozaki, N., and Soderling, T.R. (2004). Calcium activation of ERK mediated by calmodulin kinase I. J. Biol. Chem. *279*, 24064–24072.

Schmitt, J.M., Guire, E.S., Saneyoshi, T., and Soderling, T.R. (2005). Calmodulin-dependent kinase kinase/calmodulin kinase I activity gates extracellular-regulated kinase-dependent long-term potentiation. J. Neurosci. *25*, 1281–1290.

Scott, E.K., and Luo, L. (2001). How do dendrites take their shape? Nat. Neurosci. *4*, 359–365.

Soderling, T.R. (1999). The Ca^{2+}-calmodulin-dependent protein kinase cascade. Trends Biochem. *24*, 232–235.

Suizu, F., Fukuta, Y., Ueda, K., Iwasaki, T., Tokumitsu, H., and Hosoya, H. (2002). Characterization of Ca^{2+}/calmodulin-dependent protein kinase I as a myosin II regulatory light chain kinase in vitro and in vivo. Biochem. J. *367*, 335–345.

Takemoto-Kimura, S., Terai, H., Takamoto, M., Ohmae, S., Kikumura, S., Segi, E., Arakawa, Y., Furuyashiki, T., Narumiya, S., and Bito, H. (2003). Molecular cloning and characterization of CLICK-III/CaMKIgamma, a novel membrane-anchored neuronal Ca^{2+}/calmodulin-dependent protein kinase (CaMK). J. Biol. Chem. *278*, 18597–18605.

Thomas, G.M., and Huganir, R.L. (2004). MAPK cascade signalling and synaptic plasticity. Nat. Rev. Neurosci. *5*, 173–183.

Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M., and Hidaka, H. (1990). KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiper azi ne, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. J. Biol. Chem. *265*, 4315–4320.

Tokumitsu, H., Inuzuka, H., Ishikawa, Y., Ikeda, M., Saji, I., and Kobayashi, R. (2002). STO-609, a specific inhibitor of the Ca(2+)/calmodulin-dependent protein kinase kinase. J. Biol. Chem. *277*, 15813–15818.

Tokumitsu, H., Inuzuka, H., Ishikawa, Y., and Kobayashi, R. (2003). A single amino acid difference between alpha and beta Ca^{2+}/calmodulin-dependent protein kinase kinase dictates sensitivity to the specific inhibitor, STO-609. J. Biol. Chem. *278*, 10908–10913.

Tokumitsu, H., Hatano, N., Inuzuka, H., Sueyoshi, Y., Yokokura, S., Ichimura, T., Nozaki, N., and Kobayashi, R. (2005). Phosphorylation of numb family proteins: possible involvement of Ca2+/calmodulin-dependent protein kinases. J. Biol. Chem. *280*, 35108–35118.

Tucker, R.P., and Matus, A.I. (1988). Microtubule-associated proteins characteristic of embryonic brain are found in the adult mammalian retina. Dev. Biol. *130*, 423–434.

Vaillant, A.R., Zanassi, P., Walsh, G.S., Aumont, A., Alonso, A., and Miller, F.D. (2002). Signaling mechanisms underlying reversible, activity-dependent dendrite formation. Neuron *34*, 985–998.

Van Aelst, L., and Cline, H.T. (2004). Rho GTPases and activity-dependent dendrite development. Curr. Opin. Neurobiol. *14*, 297–304.

Volkmar, F.R., and Greenough, W.T. (1972). Rearing complexity affects branching of dendrites in the visual cortex of the rat. Science *176*, 1445–1447.

Wayman, G.A., Kaech, S., Grant, W.F., Davare, M., Impey, S., Tokumitsu, H., Nozaki, N., Banker, G., and Soderling, T.R. (2004). Regulation of axonal extension and growth cone motility by calmodulin-dependent protein kinase I. J. Neurosci. *24*, 3786–3794.

Wong, R.O., and Ghosh, A. (2002). Activity-dependent regulation of dendritic growth and patterning. Nat. Rev. Neurosci. *3*, 803–812.

Wu, G.Y., and Cline, H.T. (1998). Stabilization of dendritic arbor structure in vivo by CaMKII. Science *279*, 222–226.

Wu, G., Malinow, R., and Cline, H.T. (1996). Maturation of a central glutamatergic synapse. Science *274*, 972–976.

Wu, G.Y., Deisseroth, K., and Tsien, R.W. (2001a). Activity-dependent CREB phosphorylation: convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway. Proc. Natl. Acad. Sci. USA *98*, 2808–2813.

Wu, G.Y., Deisseroth, K., and Tsien, R.W. (2001b). Spaced stimuli stabilize MAPK pathway activation and its effects on dendritic morphology. Nat. Neurosci. *4*, 151–158.

Yu, X., and Malenka, R.C. (2003). Beta-catenin is critical for dendritic morphogenesis. Nat. Neurosci. *6*, 1169–1177.

Yuste, R., and Bonhoeffer, T. (2001). Morphological changes in dendritic spines associated with long-term synaptic plasticity. Annu. Rev. Neurosci. *24*, 1071–1089.

Zou, D.J., and Cline, H.T. (1999). Postsynaptic calcium/calmodulin-dependent protein kinase II is required to limit elaboration of presynaptic and postsynaptic neuronal arbors. J. Neurosci. *19*, 8909–8918.