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Calmodulin-Kinases: Modulators of Neuronal Development and Plasticity

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Abstract

In the nervous system, many intracellular responses to elevated calcium are mediated by CaM kinases (CaMKs), a family of protein kinases whose activities are initially modulated by binding Ca^{2+}/calmodulin and subsequently by protein phosphorylation. One member of this family, CaMKII, is well-established for its effects on modulating synaptic plasticity and learning and memory. However, recent studies indicate that some actions on neuronal development and function attributed to CaMKII may instead or in addition be mediated by other members of the CaMK cascade, such as CaMKK, CaMKI, and CaMKIV. This review summarizes key neuronal functions of the CaMK cascade in signal transduction, gene transcription, synaptic development and plasticity, and behavior. The technical challenges of mapping cellular protein kinase signaling pathways are also discussed.

Introduction

Neuronal physiology, ranging from neurotransmitter exocytosis to dynamic regulation of dendritic spine morphology, is modified by levels of intracellular free calcium ($[Ca^{2+}]_i$) that range between 10 to 50 nM basally to stimulated levels of low micromolar. Elevations of $[Ca^{2+}]_i$ are generally short lived (milliseconds to several minutes) due to an elaborate network of calcium buffers, pumps, channels, and exchangers in cellular membranes as well as membranes of intracellular Ca^{2+}-storage organelles (e.g., endoplasmic reticulum) (Clapham, 2007). These systems, as well as the highly polarized nature of neurons, dictate subcellular compartmentalization of $[Ca^{2+}]_i$ and their temporal fluctuations in response to a large number of extracellular cues (Blackstone and Sheng, 1999).

Elevated $[Ca^{2+}]_i$ binds to numerous proteins, from low-affinity/high-capacity buffer proteins (e.g., calbindin, calretinin) that limit Ca^{2+} diffusion to proteins of higher affinity and ion specificity (e.g., calmodulin, troponin) responsible for transducing multiple biochemical changes that mediate physiological responses to elevated $[Ca^{2+}]_I$ (Clapham, 2007). These Ca^{2+}-sensor proteins decode Ca^{2+} signals based on three properties: on-rates, Ca^{2+} affinities, and subcellular localization relative to the Ca^{2+} signal (Burgoyne et al., 2004). Among these intracellular Ca^{2+}-binding proteins, calmodulin (CaM) is unique in several respects. CaM is a

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ubiquitously expressed, dumbbell-shaped 17 kD protein with each globular end containing two helix-loop-helix "EF-hand" Ca^{2+}-binding motifs (Kd = 0.5–5 μM) connected by a flexible α helix (Chin and Means, 2000). Binding of Ca^{2+} produces a conformational change in CaM, exposing hydrophobic residues that promote interactions of the Ca^{2+}/CaM complex to numerous target proteins, thereby regulating their functionalities. Many of these Ca^{2+}/CaM targets modulate cellular signaling pathways, but other targets include structural proteins, ion channels, pumps, transcription factors, and numerous rate-limiting enzymes.

Among the signaling proteins regulated by Ca^{2+}/CaM is a family of Ser/Thr protein kinases known as CaM-kinases (CaMKs) (Figure 1). CaMKs, which are particularly abundant in brain, are activated via binding of Ca^{2+}/CaM, and they phosphorylate Ser/Thr residues in their protein substrates to alter the functionality of those proteins. Some of the CaMKs, such as myosin light chain kinase, phosphorylase kinase, and CaMKIII, are dedicated to phosphorylating a single protein substrate. CaMKIII (also referred to as eEF2-kinase) phosphorylates and inactivates eukaryotic elongation factor-2 (eEF2) (Mitsui et al., 1993), and CaMKIII is unique in that it does not contain most of the canonical motifs of classical Ser/Thr protein kinases—thus, CaMKIII belongs to a separate protein kinase family (Ryazanov et al., 1997). Multifunctional CaMKs, such as CaMKII and members of the CaM-K cascade (CaMKK, CaMKI, and CaMKIV), are present in most mammalian tissues but are highly abundant in brain, where they phosphorylate and regulate numerous protein substrates (Figure 2). CaMKII has been intensively investigated, is thought to regulate numerous functions in neurons, and has been extensively reviewed (e.g., see the five-part minireview series in J. Neurosci. 24, 8391–8415, 2004). More recent studies have established critical roles of the CaMK cascade in neuronal development, plasticity, and behavior (Figure 2), and this will be the major focus of this review.

Structures and Regulatory Mechanisms

All of the CaMKs, except CaMKIII, have similar overall domain organizations of their 50–60 kD subunits (Figure 1) (reviewed in Soderling and Stull, 2001), and crystal structures for CaMKI (Goldberg et al., 1996) and CaMKII (Rosenberg et al., 2005) have been published. CaMKII contains a unique C-terminal subunit association domain, and electron microscopy reveals that it exists as heteromeric dodecamers of α, β, γ, and δ subunits with two hexameric rings stacked one on top of the other (Gaertner et al., 2004;Rosenberg et al., 2006). This complex oligomeric structure allows for unique regulatory mechanisms and protein-protein interaction domains that are essential to its functionality, especially in paradigms of learning and memory (reviewed in Lisman et al., 2002). Activation of CaMKII by Ca^{2+}/CaM allows intramolecular autophosphorylation of several sites, including Thr286, Thr305, and Thr306. Autophosphorylation of Thr286 has two primary consequences: (1) the subsequent dissociation of bound Ca^{2+}/CaM (i.e., when $[Ca^{2+}]_i$ levels are reduced) is decreased by several orders of magnitude, thereby prolonging its activation, and (2) even after full dissociation of Ca^{2+}/CaM, the kinase retains partial (30%–60%) activity (i.e., Ca^{2+}-independent or constitutive/autonomous activity, Figure 1). Thus, transient elevations of intracellular $[Ca^{2+}]_i$ can result in prolonged CaMKII activity until protein phosphatases dephosphorylate Thr286 (Colbran, 2004a). Moreover, the extent of CaMKII autonomous activity can be dictated by the frequency of Ca^{2+} oscillations (De Koninck and Schulman, 1998). This mechanism is thought to be critical in several physiological situations, especially potentiation of synaptic transmission during learning and memory (Lisman et al., 2002). Thus, transgenic mice in which Thr286 is mutated to Ala or Asp exhibit multiple behavioral and learning deficits (see section on Synaptic Plasticity and Behavior).

Members of the CaMK cascade—CaMKK (α and β), CaMKI (α, β, δ, and γ), and CaMKIV (one gene)—are monomeric and, apart from activation by Ca^{2+}/CaM, show very different modes of regulation by phosphorylation compared to CaMKII (Figure 1). These CaMKs, like

most other Ser/Thr protein kinases, have an "activation loop" phosphorylation site that is absent in CaMKII. Binding of Ca^{2+}/CaM to CaMKI and CaMKIV exposes this activation loop site to allow phosphorylation by the upstream CaMKK when simultaneously activated by Ca^{2+}/CaM. Phosphorylation of the activation loop in CaMKI and CaMKIV primarily increases their Ca^{2+}/CaM-dependent activities. CaMKIV, but not CaMKI, can also exhibit significant Ca^{2+}-independent activity (Chatila et al., 1996;Tokumitsu et al., 2004) (Figure 1). In neurons, CaMKK-mediated phosphorylation/activation of CaMKIV appears to be quite transient, lasting for only a few minutes (Kasahara et al., 2001;Uezu et al., 2002), whereas CaMKI phosphorylation can persist up to an hour or more (Schmitt et al., 2005). Although CaMKI and CaMKIV have overlap in substrate specificity determinants (Lee et al., 1994), they can also exhibit unique phosphorylation site preferences even in the same protein substrates (Corcoran et al., 2003). Cellular substrate specificities of CaMKI and CaMKIV are often dictated by different subcellular localizations (see section on Subcellular Targeting).

The primary substrates of CaMKK are CaMKI and CaMKIV, but CaMKK can also activate PKB/Akt (Yano et al., 1998) and AMP-kinase (reviewed in Witters et al., 2006) (Figure 2). PKB and AMP-kinase are referred to as secondary substrates because their phosphorylation/activation by CaMKK is very slow. For example, activation of CaMKI upon NMDA receptor stimulation occurs in less than 5 min, whereas PKB/Akt activation by CaMKK is maximal in about 60 min (Schmitt et al., 2005). CaMKK-mediated activation of PKB upon prolonged elevation of $[Ca^{2+}]_I$ results in PKB phosphorylation and inactivation of the proapoptotic factor BAD, thereby protecting neurons from apoptosis (Yano et al., 1998) (Figure 2). AMP-kinase is critical for regulation of cellular energy metabolism in many tissues, and its function(s) in brain is just beginning to be explored. Recent evidence implicates it in hypothalamic production of neuropeptide Y and weight regulation (Anderson et al., 2008). Intriguingly, a brain-specific member of the AMP-kinase family, SAD-B, has recently been identified as a target for CaMKK-mediated phosphorylation/activation (Fujimoto et al., 2008). This may be of interest, as mammalian SAD-B is involved in neuronal polarization (Barnes et al., 2007; Kishi et al., 2005) and presynaptic neurotransmitter release (Inoue et al., 2006). The potential role of CaMKK in regulating neuronal functions via SAD-B warrants further investigation.

Subcellular Targeting of CaM-Kinases

Many protein kinases, including CaMKs (Lee et al., 1994), have significant overlap in their substrate recognition motifs, leading to a lack of substrate specificity in vitro. Cellular specificity is often obtained by colocalization of substrates with kinases (Tsui et al., 2005) through their participation in multiprotein signaling complexes or targeting to subcellular compartments such as the nucleus (nuclear import or export signals) or membranes (lipid modifications). In addition to colocalization with specific substrates, subcellular compartmentalization of CaMKs can also dictate their activation kinetics (Inagaki et al., 2000), as $[Ca^{2+}]_I$ dynamics vary considerably within the neuron (e.g., dendritic spine versus dendritic shaft) (Sabatini et al., 2001).

CaMKII is present throughout the neuron as a soluble protein, but it is also localized through interactions with multiple proteins. For example, an insert in the β subunit of CaMKII specifically binds to and bundles F-actin to promote isoform-specific neurite outgrowth (Fink et al., 2003) and maintain dendritic spines (Okamoto et al., 2007). Importantly, CaMKII constitutes the major protein of the postsynaptic density (PSD) in dendritic spines of excitatory neurons where it interacts with several proteins (reviewed in Colbran, 2004b). Interaction of CaMKII with the PSD is dynamic and regulated by binding of Ca^{2+}/CaM and the phosphorylation status of CaMKII—autophosphorylation of Thr286 promotes and stabilizes CaMKII binding to the PSD (Strack et al., 1997). Interaction with the NMDAR, predominantly with the NR2B subunit, is of particular interest since this channel has Ca^{2+} permeability and

several substrates of CaMKII exist in the PSD. Numerous studies have documented that stimulation of the NMDAR in neurons, which triggers autophosphorylation of CaMKII, promotes translocation of CaMKII to the PSD. In hippocampal neurons, a brief stimulus promotes a transient and reversible interaction of CaMKII with NR2B, whereas a stronger stimulus results in persistent translocation of CaMKII (Bayer et al., 2006). These two modes of interaction involve different domains in CaMKII—the persistent interaction elicits constitutive activity of CaMKII. Induction of LTP in rat hippocampal slices increases CaMKII bound in spines (Otmakhov et al., 2004), and the association of CaMKII with the PSD correlates with synaptic strength of individual spines (Asrican et al., 2007).

To assess the role of NMDA receptor subunit NR2B interaction with PSD molecules (e.g., α-CaMKII), a transgenic mouse expressing a C-terminal fragment of NR2B fused to a mutant form of the ligand-binding domain of the estrogen receptor (NR2B-LBD) was generated (Zhou et al., 2007). Treatment with tamoxifen to elevate NR2B-LBD disrupted the interaction between α-CaMKII and NR2B and led to deficits in LTP induction and spatial learning (Zhou et al., 2007), consistent with an earlier study (Barria and Malinow, 2005). Interestingly, activation of the transgenic protein also decreased both the activity of α-CaMKII (i.e., auto-phosphorylation at Thr286) and the phosphorylation of a key CaMKII substrate, the AMPA receptor subunit glutamate receptor 1 (Barria et al., 1997), critical for LTP induction (Zhou et al., 2007). These results showed that interaction with NR2B can modulate α-CaMKII activity, AMPA receptor function, and therefore LTP and learning.

Whereas autophosphorylation of Thr286 stabilizes binding of CaMKII with the PSD, autophosphorylation of Thr305/306 suppresses this interaction (Elgersma et al., 2002). Intriguingly, in a mouse model of Angelman syndrome, a neurological disorder associated with severe mental retardation, there is enhanced phosphorylation of Thr305 and a reduction in the amount of CaMKII associated with the PSD (Weeber et al., 2003). Since this mouse model also exhibits deficits in LTP (Jiang et al., 1998), these results are consistent with the hypothesis that PSD-associated CaMKII is critical for LTP and implicates CaMKII in mental retardation.

CaMKK is present in neurons throughout the brain where the α isoform is distributed throughout the cell, and the β isoform may also be nuclear (Nakamura et al., 2001; Sakagami et al., 2000). CaMKI is predominantly cytoplasmic (Picciotto et al., 1995), but a splice variant of the β isoform is nuclear (Ueda et al., 1999). Cytoplasmic localization of the α isoform is probably due to the presence of a nuclear export signal in its regulatory domain (Stedman et al., 2004). The γ isoform, which is lipid modified, can associate with the Golgi and plasma membranes (Takemoto-Kimura et al., 2003). Plasma membrane localization of the γ isoform is probably important for the ability of this isoform to exert crosstalk with the Ras/Erk pathway (Wayman et al., 2006), since activated Ras is also associated with the plasma membrane. Subcellular localization of CaMKK and CaMKI with lipid rafts and/or scaffold proteins is important for their physiological functions, as detailed in the section on Neuronal Development. Based on immunostaining of brain sections and analysis of cultured neurons, active CaMKIV is predominantly nuclear (Jensen et al., 1991) due to facilitated transport by importin α (Kotera et al., 2005; Lemrow et al., 2004). CaMKIV can form a complex with protein phosphatase 2A, which may account for its relatively transient activation in cells by CaMKK (Anderson et al., 2004; Kasahara et al., 2001; Westphal et al., 1998). CaMKIV is most likely involved in regulating gene transcription (see Gene Transcription).

Crosstalk between CaM-Kinases and Other Signaling Pathways

Cellular signaling pathways are seldom linear—often they form complex networks with other signaling pathways. This crosstalk can occur at several levels—there can be direct phosphorylation between protein kinases or regulatory phosphorylation of either an upstream

or downstream effector of another kinase. For example, three splice variants of CaMKII contain an NLS that targets them to the nucleus—this NLS can be phosphorylated by CaMKI or CaMKIV, thereby preventing their nuclear localization (Heist et al., 1998). CaMKII can directly phosphorylate another CaM-dependent kinase, myosin light chain kinase (MLCK) (Hashimoto and Soderling, 1990), thereby reducing binding of Ca^{2+}/CaM to MLCK and its sensitivity to Ca^{2+} (Tansey et al., 1992). As mentioned above, CaMKII directly binds the NMDAR, but it is also colocalized in the PSD with the Ras/Rap-GAP, syn-GAP, through the PDZ scaffold protein MUPP1 (Krapivinsky et al., 2004). Details of synGAP regulation by CaMKII and the downstream consequences are somewhat controversial (Chen et al., 1998; Krapivinsky et al., 2004; Oh et al., 2004; Rumbaugh et al., 2006), but it appears to modulate membrane insertion of AMPARs, dendritic spine morphology, and synaptic responses (Krapivinsky et al., 2004; Vazquez et al., 2004).

Several examples of crosstalk have been identified with the CaMKK/CaMKI cascade (Figure 2). CaMKK activity can be suppressed through PKA-mediated phosphorylation of Ser74, Thr108, and Ser458. Ser458 is within the CaM-binding domain, and its phosphorylation suppresses binding of Ca^{2+}/CaM to CaMKK (Wayman et al., 1997), whereas phosphorylation of Ser74 promotes binding of protein 14-3-3 and inhibition of CaMKK (Davare et al., 2004). Therefore, potential for a negative feedback loop exists: Ca^{2+}/CaM would rapidly activate CaMKK, whereas subsequent activation of CaM-dependent adenylate cyclase would elevate cAMP to activate PKA, thereby phosphorylating and inhibiting CaMKK.

In addition to crosstalk between the PKA and CaMKK signaling pathways, there is very important communication between the CaMK cascade and the Mek/Erk pathway (Figure 2) (Schmitt et al., 2004). Stimulation of the NMDAR in cultured neurons or hippocampal slices strongly activates Erk, a process suppressed by dnCaMKK or dnCaMKI but not by either nuclear-localized dnCaMKIV or inhibition of CaMKII (Schmitt et al., 2005). This Erk activation appears to be mediated by γCaMKI, since its RNAi knockdown, but not suppression of the other CaMKI isoforms, blocks activity-dependent Erk activation (Wayman et al., 2006). This NMDAR-dependent crosstalk between CaMKK/CaMKI and Mek/Erk appears to be important for a component of E-LTP (Schmitt et al., 2005) and in activity-dependent dendritic arborization (Wayman et al., 2006) (see sections on Synaptic Plasticity and Neuronal Development). However, the detailed mechanism whereby CaMKK/CaMKI activate MEK/Erk remains to be elucidated.

Criteria for Evaluating Physiological Functions of CaM-Kinases

Cellular signaling pathways need to be established using multiple, independent techniques, including pharmacological reagents, expression of dominant-interfering or constitutively active constructs, RNAi approaches, and targeted and/or temporally restricted gene knockouts, since each approach by itself has serious limitations. Many of the physiological functions, especially in neuronal development, that had been assigned to CaMKII were based on the ability of the pharmacological reagents KN-62 and KN-93 to block those functions. Although KN-62 and KN-93 were originally thought to be specific for CaM-KII (Tokumitsu et al., 1990), both of these reagents also suppress Ca^{2+}/CaM activation of members of the CaMK cascade (Enslen and Soderling, 1994; Mochizuki et al., 1993; Hidaka and Yokokura, 1996). These KN-type drugs also have potent effects on P2X7 receptors (Baraldi et al., 2004) and K^+ (Ledoux et al., 1999) and Ca^{2+} channels (Anderson et al., 1998). In light of the lack of specificity of KN-62 and KN-93, many of the previously reported functions of CaMKII based on studies using these inhibitors need to be reassessed in terms of potential regulation by other CaM-kinases. Indeed, recent studies, many of which are reviewed herein, have substantiated these concerns and shown regulatory roles for members of the CaMK cascade, especially in neuronal development. These results highlight the danger of assigning cellular functionality to

a signaling molecule based on any single experimental approach. It should be noted that endogenous CaMKII activity in neurons can be suppressed by RNAi (Okamoto et al., 2007) or blocked by transfection of the full-length CaMKIIN (Wayman et al., 2004) or treatment with membrane-permeable peptides (Fink et al., 2003; Sanhueza et al., 2007; Yang et al., 2004) derived from either CaMKIIN (Chang et al., 1998) or the autoinhibitory domain of CaMKII (Ishida et al., 1995). CaMKIIN is an endogenous neuronal protein that specifically inhibits CaMKII with little of no effect on other CaM-kinases, PKA, PKC, or Erk (Chang et al., 1998, 2001).

A caveat that needs to be considered when overexpressing kinases, either by transfection of cultured cells or in transgenic mice, is that overlap of substrate specificity determinants among several protein kinases can confound interpretation of results. Due to high levels of expression, the kinases may mislocalize and have access to substrates not available to their endogenous counterpart that has a discrete subcellular compartmentalization (see section on Subcellular Targeting). For example, the observation that transfected caCaMKIV can activate Erk (Enslen et al., 1996) is probably accounted for by its mislocalization outside of the nucleus due to overexpression—more recent studies clearly identify CaMKI in Erk activation (Schmitt et al., 2004, 2005; Wayman et al., 2006). This conclusion about mislocalization of expressed CaMKIV is consistent with the fact that transfected dominant-negative CaMKIV (dnCaMKIV) is expressed throughout the neuron (Lemrow et al., 2004) and suppresses axonal outgrowth (Wayman et al., 2004). However, if a nuclear localization signal is attached to restrict its expression to the nucleus and thereby mimic endogenous CaMKIV, this NLS-dnCaMKIV no longer regulates axonal elongation or activity-dependent dendritic growth that physiologically are regulated by CaMKK/CaMKI (Wayman et al., 2004, 2006). There is an additional concern when overexpressing constitutively active (ca) kinases, since they are constantly active and can overpower opposing protein phosphatases. Moreover, some studies use expression of truncated CaMKs containing only the catalytic domain that may also mislocalize. For example, full-length αCaMKI is predominantly cytoplasmic, whereas the truncated, constitutively active construct (e.g., residues 1–293) is also nuclear due to the loss of the nuclear export signal in the regulatory domain (Stedman et al., 2004). Thus, their high levels of constant activity and mislocalization can potentially yield nonphysiological results.

RNAi approaches to suppress expression of endogenous proteins have become popular, but they need to be controlled by demonstrating rescue through transfection of a homolog that is resistant to the RNAi. The major concern of the traditional gene knockout approach is that developmental deficits or compensatory expression of other isoforms may obscure physiological/behavioral responses in mature animals. For example, any of the germ line mutations studied could have affected development or other processes that could have changed the biochemistry, neurophysiology, or neuroanatomy of the brain prior to the behavioral tests administered, thus confounding the interpretation of the results. These concerns have been largely obviated by use of spatially and/or temporally restricted knockout paradigms, but even here sometimes considerable time is necessary for effects to materialize. Thus, mapping physiological functions of signaling pathways can be complex and requires corroborating results from several independent approaches. Of course, most neuronal readouts (e.g., dendritic spine formation) require numerous individual steps, each of which may be regulated in part by several signaling pathways. Therefore, it is to be expected that multiple signaling molecules are required for such multifaceted phenomena.

Regulation of Gene Transcription and Protein Synthesis

Neuronal development and plasticity are dependent on transcription of numerous genes regulated by agonists or cues that alter $[Ca^{2+}]_I$ (reviewed in Flavell and Greenberg, 2008). Several Ca^{2+}-responsive transcription factors, such as CREB and ATF-1, have been identified

in vitro and in situ as targets for CaMKs. The most intensively investigated of these is CREB, which requires phosphorylation on Ser133 to stimulate transcription (Impey and Goodman, 2001). One caveat is that multiple kinases (PKA, CaMKs, MAP-kinases), all of which can be activated by elevated $[Ca^{2+}]_I$, can phosphorylate Ser133. Therefore, sorting out which kinase mediates a particular physiological Ca^{2+}-dependent response can be difficult. Although CaMKII can phosphorylate Ser133 (Sheng et al., 1991), it also phosphorylates Ser142, which blocks CREB-mediated transcription even when Ser133 is phosphorylated (Sun et al., 1994).

Transfection with constitutively active CaMKI or CaMKIV can stimulate transcription of a CREB reporter gene (Sun et al., 1996). Of these, CaMKIV, which is nuclear localized, may be important for direct phosphorylation of CREB or its related transcription factor ATF-1 (Sun et al., 1996) (Figure 2). However, in depolarized neurons, CaMKIV may only contribute to an early phase of CREB phosphorylation, whereas prolonged CREB phosphorylation, which is required for gene transcription, appears to be mediated by the MAP-kinase pathway (Wu et al., 2001a). Indeed, induction of LTP results in a CaMKK-mediated phosphorylation and activation of nuclear CaMKIV (Kasahara et al., 2001) that is relatively transient (~10 min), probably due to associated protein phosphatase 2A (Anderson et al., 2004; Westphal et al., 1998). However, it appears that the Ca^{2+}-independent activity of CaMKIV is required for transcriptional readout (Chow et al., 2005), and this parameter has not been measured in neurons. Once phosphorylated on Ser133, CREB recruits its co-activators CBP and p300. CaMKIV can also phosphorylate CBP and thereby stimulate CREB-dependent transcription (Hardingham et al., 1999; Impey et al., 2002). In summary, the above studies, as well as those using transgenic animals (see section on Synaptic Plasticity and Behavior), on the role of CaMKIV in regulating CREB/CBP-mediated gene transcription are suggestive but not conclusive at this time.

Although a splice variant of the β isoform of CaMKI is nuclear, its role in direct stimulation of gene transcription has not been tested. However, CaMKI does contribute strongly to Ca^{2+}-mediated transcription in neurons through crosstalk with the Ras/Erk pathway (Schmitt et al., 2004). In cultured hippocampal neurons or acute slices, NMDA-stimulated activation of Erk is predominantly mediated through CaMKK/CaMKI (Schmitt et al., 2005). Furthermore, this pathway appears to be important for dendritic arborization where activity-dependent NMDAR activation of the γ isoform of CaMKI results in MEK/Erk-mediated CREB-regulated transcription of Wnt-2 (Wayman et al., 2006) and microRNA132 (Wayman et al., 2008) (see section on Neuronal Development).

Most dendritic proteins are translated in the soma and subsequently transported into the dendrites. However, the mRNA for some synaptic proteins (including α-CaMKII) is itself transported to dendrites and translated near or within postsynaptic spines (Mayford et al., 1996b). Blocking the dendritic translocation of α-CaMKII mRNA by targeted deletion of its 3′UTR resulted in a profound (>75%) reduction in synaptic (i.e., PSD associated) α-CaMKII protein, as well as deficits in both the stability of L-LTP and memory. Unlike the L-LTP and long-term memory, the E-LTP and short-term memory were unaffected by this mutation (Miller et al., 2002). These remarkable results suggest that the local translation and subcellular localization of α-CaMKII at synaptic sites may be important for the stability, but not induction, of LTP and for memory but not learning.

Protein synthesis itself is also subject to regulatory phosphorylation. There are several proteins that enhance cap-dependent mRNA initiation, such as mTOR, eIF4E, and its inhibitory binding protein 4E-BP1, that are positively regulated by phosphorylation, in part via MAP-kinases (Wang et al., 1998). Induction of LTP enhances phosphorylation of these factors (Kelleher et al., 2004; Schmitt et al., 2005), and mice expressing dnMEK1 show an impairment in L-LTP and in phosphorylation of these translation factors (Kelleher et al., 2004). These

phosphorylations may be mediated by the CaMKK/CaMKI crosstalk with MEK/Erk, since treatment with STO-609 blocks their phosphorylation in response to LTP induction (Schmitt et al., 2005). However, corroboration by independent approaches is needed.

In addition to general translation factors, there are several proteins that regulate translational initiation of specific mRNA species through binding to specific elements in the 3′UTR. One of these, cytoplasmic polyadenylation element binding protein 1 or CPEB1 (Richter, 2007), is part of a multiprotein complex containing Maskin and eIF4E that regulates translation of proteins (e.g., αCaMKII, insulin receptor substrate protein 53, tissue plasminogen activator, AMPA receptor binding protein) (Bramham and Wells, 2007), several of which are involved in synaptic development and/or plasticity. CPEB1 promotes the transport of selected mRNAs into dendrites where they undergo activity-dependent polyadenylation and translation (Huang et al., 2002; Wu et al., 1998). CPEB1, upon phosphorylation of Thr171 and/or Ser177 by Aurora kinase, promotes polyadenylation of these mRNAs and releases eIF4E from its inhibitory interaction with Maskin, thereby stimulating translation (Richter, 2007). Based on activity-dependent polyadenylation in neurons, several other proteins may be regulated by CPEB (Du and Richter, 2005). CaMKII can also phosphorylate CPEB1 and stimulate synthesis of CPE-containing mRNAs (Atkins et al., 2004). Induction of chemical LTD promoted transient dephosphorylation of CPEB1, whereas induction of L-LTP, but not E-LTP, resulted in CaMKII-dependent phosphorylation of CPEB1 that was prolonged due to simultaneous inhibition of protein phosphatase 1 (PP1) (Atkins et al., 2005). These results suggest that certain paradigms of Ca^{2+}-mediated synaptic plasticity modulate the phosphorylation status of CPEB1 through CaMKII and PP1 to regulate protein synthesis in dendrites.

In contrast to enhanced translational initiation by CaMKs as discussed above, translational elongation of most proteins is suppressed via phosphorylation of eEF2 by CaMKIII (also known as eEF2-kinase) (Mitsui et al., 1993). This CaMKIII/eEF2 pathway appears to be critically involved in LTD by group 1 metabotropic receptors (mGluRs) and requires the immediate-early gene Arc/Arg3.1 (Park et al., 2008). CaMKIII is bound to mGluRs, in part through an interaction with the PSD scaffold protein Homer, but this interaction is reduced by mGluR activation and Ca^{2+} elevation. Paradoxically, synthesis of Arc/Arg3.1, which promotes AMPAR endocytosis through interaction with endophilin 2/3 and dynamin (Chowdhury et al., 2006), is rapidly (5 min) enhanced by CaMKIII phosphorylation of eEF2 (Park et al., 2008). Thus, genetic deletion of CaMKIII results in a selective loss of rapid mGluR-dependent Arc/Arg3.1 translation and in LTD induced by either mGluR stimulation or paired-pulse low-frequency stimulation but not in NMDAR-dependent LTD or E-LTP. This pathway appears to also have implications for synaptic plasticity in fragile X syndrome, the most common inherited cause of mental retardation and autism (O'Donnell and Warren, 2002). The fragile X mental retardation protein (FMRP) that is reduced in this syndrome is a translational repressor of specific synaptic mRNAs. In FMRP knockout mice, there is an increased association of Arc/Arg3.1 with polyribosomes, presumably resulting in enhanced basal synthesis of Arc/Arg3/1 to account for the fact that mGluR LTD in these mice is protein synthesis independent (Hou et al., 2006; Nosyreva and Huber, 2006).

Neuronal Development

Several aspects of neuronal development, such as outgrowth and pathfinding of axons and dendritic arborization and spine formation, are regulated in part by localized Ca^{2+} dynamics (Konur and Ghosh, 2005; Zheng and Poo, 2007). Many of the stimulatory effects of Ca^{2+} were ascribed to CaMKII because they were largely suppressed by KN-62 or KN-93 (Kuhn et al., 1998; Solem et al., 1995; Vaillant et al., 2002; Williams et al., 1995), originally thought to be specific inhibitors of CaMKII (Tokumitsu et al., 1990). With the realization that these pharmacological compounds inhibit other targets, including CaMKI and CaMKIV (Enslen and

Soderling, 1994; Hidaka and Yokokura, 1996; Mochizuki et al., 1993), roles of other CaMKs in neuronal development have also been examined.

These recent studies show that multiple members of the CaMK family perform essential functions in the development of neurons. Activation of CaMKII in *Xenopus* optic tectum reduces the rate of dendritic outgrowth through stabilizing the dendritic arbor late in development (Wu and Cline, 1998; Zou and Cline, 1999). In depolarized hippocampal neurons, βCaMKII exerts a stimulatory effect on filopodial motility early in development (3 DIV) but, as in the *Xenopus* optic tectum (Wu and Cline, 1998), is inhibitory at later times (11 DIV) when synaptogenesis is prominent (Fink et al., 2003). CaMKII has also been implicated in regulating Ca^{2+}-dependent axonal growth cone attraction (Wen et al., 2004). Relatively large local Ca^{2+} transients activate CaMKII to give attraction, whereas smaller Ca^{2+} signals mediate repulsion through the CaM-dependent phosphatase calcineurin. CaMKII also can regulate dendritic spines through at least two mechanisms. As mentioned previously, βCaMKII binds and bundles F actin, and RNAi knockdown of β, but not α, CaMKII reduces the number of mature spines in cultured hippocampal slices (Okamoto et al., 2007). This effect of βCaMKII does not require kinase activity, as a kinase-dead mutant is able to rescue the RNAi effect. CaMKII also modulates enlargement of dendritic spines and recruitment of AMPARs through its phosphorylation and activation of the Rac1 GEF kalirin-7 (Xie et al., 2007) (Figure 3).

Depolarization of cortical neurons (2–4 DIV) promotes dendritic growth that requires activation of both ERK and a CaMK to stimulate CREB-mediated transcription (Redmond et al., 2002). The authors concluded that this CaMK is CaMKIV because expression of caCaMKIV phenocopies the effect of depolarization on dendritic outgrowth. Two very recent articles have likewise concluded involvement of CaMKIV via CREB in dendritic development and synapse formation based on transfection of either dnCaMKIV or caCaMKIV (Tai et al., 2008; Zhou et al., 2008). However, as discussed above (Criteria for Physiological Functions), transfected CaMKIV is not nuclear restricted (Wayman et al., 2004), like endogenous CaMKIV (Jensen et al., 1991), and therefore it can mimic cytosolic CaMKI, which activates Erk (Schmitt et al., 2004) and CREB-dependent transcription (Wayman et al., 2006). When a nuclear localization signal is attached to restrict transfected dnCaMKIV expression to the nucleus, this dnCaM-KIV does not suppress axonal outgrowth or activity-dependent dendritic arborization that are actually mediated by CaMKI (Wayman et al., 2004, 2006). However, since there may be some nonnuclear CaMKIV in neurons, it will be important to determine whether reduction of endogenous CaMKIV using RNAi approaches, as has been done for CaMKI (see below), influences neuronal development. CaMKIV knockout mice apparently have normal forebrain development with some impairment of Purkinje development in cerebellum (Ribar et al., 2000). In another study, neuronal activity, mimicked by repetitive KCl depolarizations, stimulates filopodial formation on hippocampal neuron dendrites that involves both Erk and a CaMK (Wu et al., 2001b). This is presumably mediated by CaMKK/CaMKI activation of Erk (Schmitt et al., 2004; Wayman et al., 2006).

More recent studies have directly tested roles of CaMKK/CaMKI in neuronal development using multiple, independent approaches: pharmacological inhibitors, transfection of dominant-negative constructs, and suppression of endogenous signaling molecules using RNAi. In most of these studies (Saneyoshi et al., 2008; Wayman et al., 2004, 2006), inhibition of neuronal development by the CaMKK inhibitor STO-609 was rescued by transfection with a mutant of CaMKK that is 100-fold less sensitive to inhibition by STO-609 (Tokumitsu et al., 2003). Likewise, inhibitory effects of RNAi against the rat homologs were rescued by human homologs. These series of studies, performed largely with cultured neurons but with many results confirmed in brain slices, have demonstrated requirements for CaMKK/CaMKI in regulation of axonal growth cone morphology/motility and axonal outgrowth (Wayman et al., 2004), dendritic arborization (Takemoto-Kimura et al., 2007; Wayman et al., 2006, 2008), and

formation of dendritic spines and synapses (Saneyoshi et al., 2008) (Figures 3 and 4). Initial outgrowth of dendrites (2 DIV, total length ~100 μm) in immature cortical neurons involves CaMKIγ—over-expression of CaMKIγ enhances dendritic length, whereas RNAi against this isoform suppresses outgrowth and branching by ~50% (Takemoto-Kimura et al., 2007). Furthermore, cultured cortical neurons from a CaMKIγ knockout mouse exhibit about a 25% reduction in dendritic length and branching. Importantly, the CaMKIγ is colocalized in lipid rafts with Rac and its GEF, STEF (Figure 4). Expression of a dominant-negative STEF blocks neurite enhancement by overexpressed CaMKIγ. Effects of RNAi against other CaMKI isoforms was not investigated in this study nor was the mechanism by which CaMKIγ activates STEF. This same signaling pathway also mediates Ca^{2+}-dependent stimulatory effects of BDNF on early dendritogenesis (Takemoto-Kimura et al., 2007). In another study using hippocampal neurons (Wayman et al., 2006), basal dendritogenesis from 4–9 DIV was not affected by the CaMKK inhibitor STO-609 under conditions where axonal outgrowth was strongly suppressed (Wayman et al., 2004). However, activity-enhanced dendritic arborization in mature neurons (7–9 DIV, total dendritic length ~1200 μm) was dependent on NMDAR activation of both the α and γ isoforms of CaMKI (Wayman et al., 2006). The CaMKIγ effect was mediated via crosstalk to activate MEK/Erk (Schmitt et al., 2004) and subsequent CREB-dependent transcription of Wnt-2 (Wayman et al., 2006) and microRNA132 (Wayman et al., 2008) (Figure 4). Previous studies have demonstrated that Wnts, via interaction with β-catenin, enhance dendritic arborization (Yu and Malenka, 2003). Conversely, microRNA132 functions by suppressing the expression of p250GAP, a Rac/Cdc42 GAP. The consequence of decreased p250GAP levels is an increase in Rac activity and stimulated dendritic growth (Wayman et al., 2008). It is intriguing that different temporal aspects of dendritic development may be mediated by CaMKIγ utilizing different signaling pathways—BDNF enhancement of early development is via lipid raft CaMKIγ activation of STEF/Rac to modify the actin cytoskeleton (Takemoto-Kimura et al., 2007), whereas subsequent activity-dependent arborization is mediated in part by MEK/Erk/CREB transcription of Wnt-2 and microRNA132 (Wayman et al., 2006, 2008) (Figure 4). It will be important to determine whether different sources of intracellular Ca^{2+} elevation (e.g., BDNF versus NMDAR) activate different subcellular pools or isoforms of CaMKI. A current limitation in these studies is the lack of isoform-specific antibodies. CaMKIα also plays a role in activity-dependent dendritic arborization through an unidentified pathway (Wayman et al., 2006). Potential candidates (see Table 1) include regulation of the actin cytoskeleton via CaMKI-mediated phosphorylation of myosin II regulatory light chain (Suizu et al., 2002) or via Rac1 activation (Saneyoshi et al., 2008). Another possibility is CaMKI phosphorylation of Numb (Tokumitsu et al., 2005, 2006), which regulates neuronal development (Nishimura et al., 2003, 2006).

Recent work demonstrates that CaMKK and CaMKI modulate another very important aspect of neuronal development—formation of dendritic spines and synapses (Saneyoshi et al., 2008). CaMKK and CaMKIα are colocalized with βPIX and GIT1 in dendritic spines as part of a multiprotein complex. Since βPIX is a Rac/Cdc42 GEF and one of the major effectors of Rac is Pak1, which also binds βPIX, βPIX coordinates Rac-dependent activation of Pak1 (Mott et al., 2005). There are numerous downstream effectors of Pak1 that regulate actin dynamics (Bokoch, 2003). Subcellular localization of βPIX to the postsynaptic density of dendritic spines is mediated largely by scaffold proteins such as GIT1 (Ko et al., 2003; Zhang et al., 2003) and Shank (Park et al., 2003). Endogenous neuronal activity, via NMDAR stimulation of CaMKK and CaMKI, promotes αCaMKI phosphorylation of βPIX to enhance GTP loading of Rac1 and developmental formation of mushroom-shaped spines and synapses (Saneyoshi et al., 2008) (Figure 3).

The CaMK cascade may also regulate neuronal development through modulation of microtubules. Upon activation by Ca^{2+}/CaM, the catalytic domain of CaMKI interacts with and phosphorylates multiple sites on microtubule affinity regulating kinase 2 (MARK2)

(Uboha et al., 2007). MARK2, which is downstream of the PAR-3/PAR-6/PKC complex, promotes axon specification through regulation of the microtubule cytoskeleton (Chen et al., 2006). Enhanced axonal elongation due to overexpression of ca-CaMKI is blocked by coexpression of the MARK2 mutant where the CaMKI sites have been mutated (Uboha et al., 2007). Microtubule dynamics are also regulated by stathmin phosphorylation on Ser16 induced by overexpression of caCaMKIV (Melander Gradin et al., 1997). However, it is questionable whether endogenous nuclear CaMKIV catalyzes this reaction, but CaMKI can phosphorylate Ser16 in stathmin in vitro (G.A.W. and T.R.S., unpublished data).

Synaptic Plasticity and Behavior

There are multiple mechanisms that can produce synaptic potentiation or depression throughout the brain, but the focus here will be the CA1 synapse in the hippocampus where long-term potentiation (LTP) and depression (LTD), cellular models of learning and memory, have been most intensively investigated (reviewed in Derkach et al., 2007; Lisman et al., 2002; Luscher et al., 2000). LTP can be induced at the CA1 synapse in acute hippocampal slices by multiple induction paradigms, including high-frequency stimulation (HFS), theta-burst stimulation (TBS), and pairing of pre- and postsynaptic depolarizations (pairing-induced). The TBS protocol is probably the most physiological, as it mimics hippocampal firing patterns during active exploration and learning in rodents (Feder and Ranck, 1973; Otto et al., 1991). LTP is often divided into an early stage (E-LTP) lasting ~1 hr that does not require gene transcription and a late-phase (L-LTP, 1–4 hr) that is dependent on gene transcription, particularly mediated by the transcription factor CREB (see Gene Transcription). Induction of LTP by these multiple protocols is dependent on transient stimulation of the NMDA receptor (NMDAR) with resultant Ca^{2+} influx. However, there appear to be multiple cellular mechanisms downstream of the Ca^{2+} signal, even for E-LTP, that are mediated in part by CaMKs and result in potentiation of AMPAR currents. For example, these different mechanisms can be partially dissected using GluR1 knockout mice and/or infusion of Ca^{2+} buffers with different chelating properties into the postsynaptic neuron (Hoffman et al., 2002). HFS LTP in neonatal rodent hippocampus appears to require a PKA signaling pathway, whereas, in adult hippocampus, CaM-KII is essential (Yasuda et al., 2003). Furthermore, some potentiated neurons show an increase in unitary conductance whereas others do not (Benke et al., 1998), indicative of different mechanisms. It is likely that there are multiple Ca^{2+} sensors (e.g, CaMKI, CaMKII, adenylate cyclase, etc.) that trigger discrete downstream signaling pathways that alter synaptic trafficking of AMPARs, modulation of existing AMPAR properties (e.g., open probability, conductance), or synthesis of AMPAR subunits. Of course, even a single transducer could mediate different responses depending on its localization. For example, CaMKII bound to the NMDAR would experience Ca^{2+} dynamics and may selectively phosphorylate colocalized substrates different than soluble CaMKII in the dendritic spine head. While this review focuses on roles of CaMKs in LTP, it is clear that other Ser/Thr kinases (e.g., PKA, PKC, MAP-kinases) and tyrosine kinases (Src, Fyn) also are important, especially in L-LTP (Smolen et al., 2006).

CaMKII has unique structural and biochemical properties (see Structure and Regulatory Mechanisms) that make it an ideal candidate to be involved in LTP induction.

1. Intramolecular autophosphorylation of Thr286 allows a transient Ca^{2+} influx through the NMDAR during LTP induction to generate a sustained output signal via autonomous CaMKII activity (Figure 5).
2. The extent of holoenzyme autophosphorylation, and therefore autonomous activity, can decode the frequency of postsynaptic Ca^{2+} oscillations due to neuronal activity.
3. CaMKII is abundant in the PSD where it binds several proteins, including the NMDAR, in an activity-dependent manner (see section on Subcellular Targeting).

4. CaMKII phosphorylates several proteins in the PSD during LTP, such as AMPARs, that are thought to be important in synaptic potentiation.

Indeed, a large body of diverse evidence from molecular, cellular, and transgenic animal studies establishes CaMKII as a key player in mediating induction and expression of E-LTP by perhaps modulating trafficking and properties of AMPARs. Some of these key observations are summarized as follows.

1. Enhancing postsynaptic CaMKII activity induces synaptic potentiation that occludes subsequent induction of LTP, indicative of common mechanisms (Lledo et al., 1995).
2. Pharmacological or genetic inhibition of CaMKII activity or autophosphorylation block LTP induction (Malinow et al., 1989; Silva et al., 1992b).
3. Blocking postsynaptic CaMKII activity after induction of LTP suppress expression of LTP (Sanhueza et al., 2007).

The roles of αCaMKII in E-LTP based on molecular and cellular studies have been extensively reviewed (Colbran and Brown, 2004; Lisman et al., 2002; Malenka and Nicoll, 1999; Soderling, 2000), and results from genetic manipulations substantiate and extend these conclusions. In spite of the caveats noted previously for transgenic/knockout studies (see Criteria for Evaluating Physiological Functions), the striking consistency and convergence of the results presented with a wide variety of tools make an overwhelming case for a role for CaMKII in learning and memory. Furthermore, the evidence for its involvement in learning and memory goes well beyond the behavioral experiments described, since there is also extensive evidence for the involvement of this kinase in synaptic plasticity, and synaptic plasticity is central to learning and memory. Thus, despite the limitations of the individual experiments described herein, collectively, the data presented demonstrate that CaMKII has a key role in synaptic plasticity, learning, and memory.

The α-CaMKII gene was one of the first to be implicated in both LTP and learning and memory: deletion of α-CaMKII impaired CA1 LTP and spatial learning in mutant mice (Silva et al., 1992a, 1992b). Mice with a point mutation that prevents autophosphorylation at Thr286 were severely impaired in both LTP and spatial learning, indicating the importance of autonomous activity of this kinase for synaptic plasticity and learning/memory (Giese et al., 1998). This was extended by using a tetracycline-controlled transactivator (tTA) system to control the onset of expression of the constitutively active (T286D) α-CaMKII in mouse forebrain (Mayford et al., 1996a). These results indicated that even deregulation of this kinase in mature animals causes abnormal synaptic plasticity and learning/memory, providing evidence that the learning deficits associated with α-CaMKII mutations were not caused by developmental changes (Mayford et al., 1996a). Interestingly, not all learning and/or memory tasks were equally affected by these CaMKII mutations, indicating that not all forms of memory are supported by the same molecular synaptic mechanisms (Bach et al., 1995; Mayford et al., 1996a; Mizuno and Giese, 2005). Interestingly, α-CaMKII autophosphorylation at Thr286 is essential for NMDAR-dependent LTP (Silva et al., 1992b) in CA1 but not in the dentate gyrus (Cooke et al., 2006), suggesting that while autophosphorylation of this kinase is critical for spatial learning (Giese et al., 1998) it may not be essential for behavioral functions associated with the dentate gyrus, such as pattern separation.

αCaMKII also appears to modulate memory consolidation— this was first systematically studied with mice heterozygous for a null mutation of this kinase (Frankland et al., 2001, 2004). Memory consolidation studies revealed that both spatial learning and contextual conditioning tested 24 hr after training were unaffected by this mutation but that memory tested at later time points, including 36 days after training, was dramatically disrupted. Perhaps

because the kinase is far more abundant in the hippocampus than in the cortex, this heterozygous mutation was shown to disrupt cortical LTP without affecting hippocampal CA1 LTP (Frankland et al., 2001). Thus, these data are in agreement with the idea that plasticity mechanisms are critical for permanent memory storage in cortical networks (Frankland and Bontempi, 2005; Wiltgen et al., 2004). Current models propose that although spatial and contextual information storage may initially be dependent on hippocampal circuits, eventually this information is stored in cortical networks (Frankland and Bontempi, 2005; Wiltgen et al., 2004). Accordingly, although α-CaMKII heterozygous null mutants revealed normal hippocampal activation (i.e., immediate-early gene expression) immediately after training and following a retrieval test 24 hr later, they failed to activate cortical networks required for remote memory retrieval (i.e., 36 days after training) (Frankland et al., 2004). It is possible that the loss of cortical LTP in these mutants prevents cortical memory storage and therefore is responsible for their remote memory deficits. These results revealed the first molecular insights into remote memory storage in cortical networks: they demonstrate a correlation between αCaMKII-dependent cortical LTP and memory consolidation.

The role of CaMKII in either consolidation or retrieval of memory was investigated in more detail with an elegant chemical-genetic approach (Alaimo et al., 2001) in which the enzymatic activity of a transgenic αCaMKII was controlled by a synthetic inhibitor designed to exclusively bind the altered ATP domain of the transgenic kinase (Wang et al., 2003). With this approach, the overexpressed transgenic αCaMKII can be selectively and quickly inactivated with the synthetic inhibitor. These studies showed that elevating αCaMKII activity (by removing the synthetic inhibitor from the drinking water) resulted in dramatic changes in both LTD and LTP: LTP was generally enhanced, and stimulation parameters that normally induce LTD in the mutants induced LTP instead. Not surprisingly, the transgenic mice showed a number of deficits in learning and memory tasks that could be avoided by inhibiting the transgenic kinase prior to training and testing. Interestingly, elevating αCaMKII activity in the first week (but not in the second, third, or fourth week) following conditioning disrupted memory, suggesting that αCaMKII following training is required for memory consolidation. However, it is also possible that overexpression of this promiscuous kinase may have resulted in unphysiological phosphorylation events that affected consolidation.

Intriguingly, βCaMKII, which has different subcellular localization (e.g., bound to F actin) and Ca^{2+} responsiveness (De Koninck and Schulman, 1998), regulates synaptic transmission quite differently than the α isoform. First, the ratio of α/β CaMKII in neurons is modulated by prolonged neuronal activity that may be important in adaptation during synaptic homeostasis —increased activity favors α whereas decreased activity enhances β (Thiagarajan et al., 2002). Furthermore, αCaMKII increases unitary synaptic transmission whereas β diminishes it. It will be intriguing to determine whether the effects of βCaMKII require its catalytic activity or are a consequence of its binding to and bundling F actin and alteration of spine structure (see section on Neuronal Development).

Induction of HFS LTP results in rapid activation of nuclear CaMKIV that only persists for ~10 min (Kasahara et al., 2001) (Figure 5), probably due to its interaction with protein phosphatase 2A (Anderson et al., 2004; Westphal et al., 1998). However, studies using transgenic and knockout mice are consistent with a role for CaMKIV in LTP. Initial studies of CaMKIV knockout mice revealed impairments in CREB phosphorylation, the induction of hippocampal LTP, and in the late phase of cerebellar LTD (Ho et al., 2000; Ribar et al., 2000). A follow up study showed that long-term fear memory (tested 1 and 7 days after training) was impaired in this knockout mouse, while short-term memory (tested 1 hr posttraining) was not affected (Wei et al., 2002). Accordingly, the mutants showed substantially lower CREB activation following fear conditioning, suggesting that deficits in CREB-dependent transcription (Bourtchuladze et al., 1994) could account for their long-term memory impairments.

Confirming previous findings, however, studies in the amygdala and in multiple cortical areas revealed impairments in the induction of LTP; the behavioral results would have predicted deficits in the maintenance of LTP since long-term, but not short-term, memory was affected. It is possible that the lower levels of LTP in these mice is sufficient to support memory at 1 hr, but not at 1 or 7 days.

Kang and colleagues overexpressed a dominant-negative form of CaMKIV (dnCaMKIV) in the mouse forebrain (Kang et al., 2001). This transgenic mouse was impaired in the hidden platform version of water maze, and importantly, they showed deficits in contextual fear conditioning when tested 7 days, but not 1 day, after training. These results are surprising since the mice showed deficits in the L-LTP that could already be detected 3 hr after induction. It is possible that this difference in the time course of the memory and LTP deficits reflects the fact that these authors studied LTP in hippocampal slices. It is possible that the decline in synaptic potentiation is much slower in vivo. Interestingly, transgenic mice overexpressing CaMKIV in the forebrain (Wu et al., 2008) showed enhancements in both trace fear memory, which is known to be cortex dependent (Han et al., 2003), and in LTP in the ACC. Although there are some incongruities concerning the effects of CaMKIV manipulations, the data presented above suggest that, while decreases in the levels of CaMKIV impair LTP and memory, overexpression of this molecule can enhance LTP and memory. However, one must interpret with caution results obtained from CaMKIV overexpression studies, since it may not localize properly to the nucleus.

Transgenic and knockout studies have also implicated roles for CaMKK in plasticity and learning and memory. Effects of CaMKK deletion could result from either suppressed CaMKIV-mediated phosphorylation of CREB/CBP or via crosstalk of CaMKI with the MEK/Erk pathway and subsequent regulation of CREB-dependent transcription or other downstream targets of Erk. Deletion of the gene encoding CaMKKβ caused deficits in CREB activation and impaired L-LTP, but not E-LTP (Peters et al., 2003). Behavioral analysis indicated that this mutant has deficits in long-term, but not short-term, memory for social transmission of food preferences. Accordingly, this mutation also affected spatial learning and memory. Surprisingly, fear conditioning was unaffected in the mutants. In contrast, deletion of CaMKKα resulted in deficits in fear conditioning (Blaeser et al., 2006; Mizuno et al., 2006). After fear conditioning, the activation of both CaMKIV and CREB were significantly reduced in the hippocampus of the null mutant mice, suggesting that the CaMKKα-CaMKIV- CREB signaling cascade is involved in fear conditioning. Unlike CaMKKβ null mice, however, CaMKKα knockout mice did not show deficits in spatial learning (Mizuno et al., 2006). Surprisingly, the phenotype in both knockout mice appeared to have sex-specific components (Mizuno et al., 2006, 2007)! Male but not female CaMKKβ null mutants were impaired in CA1 LTP, and transcriptional analysis revealed sex-specific transcriptional changes (Mizuno et al., 2007). Additionally, male (but not female) CaMKKα mutant mice showed deficits in contextual conditioning- dependent transcription of BDNF (Mizuno et al., 2006). These studies demonstrate sex-specific changes in CaMKK signaling regulating plasticity and memory.

Recent molecular and cellular studies indicate a role for CaMKK/CaMKI in E-LTP via crosstalk with MEK/Erk (Schmitt et al., 2005). This effect is not transcriptional, since E-LTP does not require gene transcription. TBS induction of LTP activates Erk, and inhibitors of the Erk (e.g., U0126) pathway partially (~50%) suppress hippocampal CA1 E-LTP induction (Selcher et al., 2003). NMDAR activation of MEK/Erk appears to be mediated primarily through theCaMKK/CaMKI pathway. For example, treatment of cultured hippocampal neurons withNMDAactivates Erk—this is blocked by transfection with dominant-negative constructs of CaMKK and CaMKI but not by dominant-negative CaMKIVnuc or a specific inhibitor of CaMKII (CaMKIIN) (Schmitt et al., 2005). TBS LTP also results in a rapid (<5 min) and prolonged (>60 min) activation of CaMKI (Figure 5) and Erk, both of which are

blocked by the CaMKK inhibitor STO-609 (Schmitt et al., 2005). STO-609 inhibition of NMDAR-mediated activation of Erk is rescued by transfection with an STO-insensitive mutant of CaMKK, indicating that the STO-609 is acting via inhibition of CaMKK. TBS LTP is suppressed ~50% by inhibition of MEK/Erk (U0126) or CaMKK (STO-609), and the two effects are occlusive, suggesting that they utilize a common pathway. However, LTP induced by HFS does not appear to involve CaMKK, as it is not suppressed by STO-609 (Guire et al., 2008).

CaMKK/CaMKI may partially regulate LTP through synaptic trafficking of a special class of AMPARs, calcium-permeable AMPARs (CP-AMPARs) that lack the edited-GluR2 subunit (Isaac et al., 2007). Important roles for CP-AMPARs are established for several forms of plasticity at various brain regions. For example, replacement of basal CP-AMPARs with GluR2- containing AMPARS can account for LTD at cerebellar stellate synapses and mossy fiber-CA3 pyramidal cell synapses (Ho et al., 2007; Liu and Cull-Candy, 2000, 2005). Furthermore, synaptic localization of CP-AMPARs in various regions of the CNS is modulated by neuronal activity (Thiagarajan et al., 2005), neuronal insults (e.g., ischemia), and drugs of abuse and are implicated in several neuropathologies (reviewed in Kwak and Weiss, 2006; Liu and Zukin, 2007). However, roles of CP-AMPARs at the CA1 synapse of hippocampus are controversial. At these synapses, AMPARs are largely heteromeric complexes of GluR1 and edited-GluR2 subunits (Craig et al., 1993; Geiger et al., 1995; Monyer et al., 1991; Wenthold et al., 1996). Thus, application of CP-AMPAR inhibitors (philanthotoxin-433, IEM-1460) has little effect on basal CA1 synaptic transmission (Adesnik and Nicoll, 2007; Guire et al., 2008; Ju et al., 2004; Plant et al., 2006). However, upon induction of LTP there is a rapid but transient incorporation of synaptic CP-AMPARs (Guire et al., 2008; Plant et al., 2006) (but see Adesnik and Nicoll, 2007; Gray et al., 2007). Insertion of only a few (~5) homomeric GluR1 CP-AMPARs per synapse can significantly enhance synaptic transmission (Guire et al., 2008) due to the higher conductance of these channels and their additional potentiation by CaMKII-mediated phosphorylation of Ser831 compared to GluR2-containing AMPARs (Derkach et al., 1999; Oh and Derkach, 2005). Insertion of synaptic CP-AMPARs appears to be mediated by CaMKK/CaMKI since (1) the CaMKK inhibitor STO-609 blocks their appearance during TBS LTP and (2) infusion of activated CaMKI into cultured hippocampal neurons promotes synaptic localization of CP-AMPARs (Guire et al., 2008). This mechanism occurs with theta-burst induction (Guire et al., 2008), which is probably the most physiologically relevant protocol for LTP (Feder and Ranck, 1973; Otto et al., 1991), but not with high-frequency stimulation (Adesnik and Nicoll, 2007; Gray et al., 2007; Guire et al., 2008). Whether pairing-induced LTP utilizes CP-AMPARs is controversial (Adesnik and Nicoll, 2007; Gray et al., 2007; Plant et al., 2006).

Future Directions

Investigations over the past several years have reinforced important roles of CaMKII in synaptic plasticity and behavior. However, other studies have challenged some functions of CaMKII, based on results from using KN-62 and KN-93, in neuronal development and strongly implicate regulatory functions of the CaMK cascade, especially CaMKI. Thus, CaMKI has transitioned from an “orphan” kinase of unknown physiological function to a significant player in neuronal development and perhaps synaptic plasticity. This evolving view of neuronal functions of the various CaMKs implicates important areas for future investigation. Included in this list are the following.

1. Identification of additional multiprotein complexes that include CaMKs and give rise to subcellular signaling specificity. These studies have contributed strongly to our understanding of CaMKII functions and will continue to expand our knowledge of isoform-specific signaling specificity for other CaMKs, especially CaMKI.

2. Relationships between CaMKs and other signaling pathways. Protein kinases generally do not operate as independent, linear pathways but are incorporated into signaling networks. For example, identification of "crosstalk" between CaMKK/CaMKI and the MEK/Erk pathway has challenged our understanding of CREB-dependent transcriptional regulation by the CaMK cascade that previously was thought to be mediated solely by CaMKIV.

3. Role of CaMKIV in CREB-dependent transcription. While CaMKIV phosphorylates CREB and CBP in physiological paradigms, phosphorylation alone does not necessarily correlate with transcription. What are the relative roles of CaMIV versus other CREB-kinases, including CaMKI-mediated activation of MEK/Erk, or CBP-kinases in regulating transcription.

4. Great progress has been made in understanding roles of the various CaMKs, especially CaMKI, in neuronal development, including axonal outgrowth, dendritic maturation, and formation of spines and synapses. However, much remains to be investigated—for example, roles of CaMKI isoforms in axon formation and downstream signaling pathways regulating growth cone motility and the relative roles of CaMKI and CaMKII in temporal stages of dendritic arborization. One general limitation is the ability to strongly suppress CaMKII in neurons due to its extremely high levels of expression.

5. The functions of CaMKI and CaMKIV in long-term potentiation need to be expanded. Under what conditions and by what mechanisms does CaMKI mediate transient synaptic incorporation of calcium-permeable AMPARs into synapses? What unique functions do these receptors serve? Is CaMKIV-mediated CREB-dependent transcription important for L-LTP and memory consolidation?

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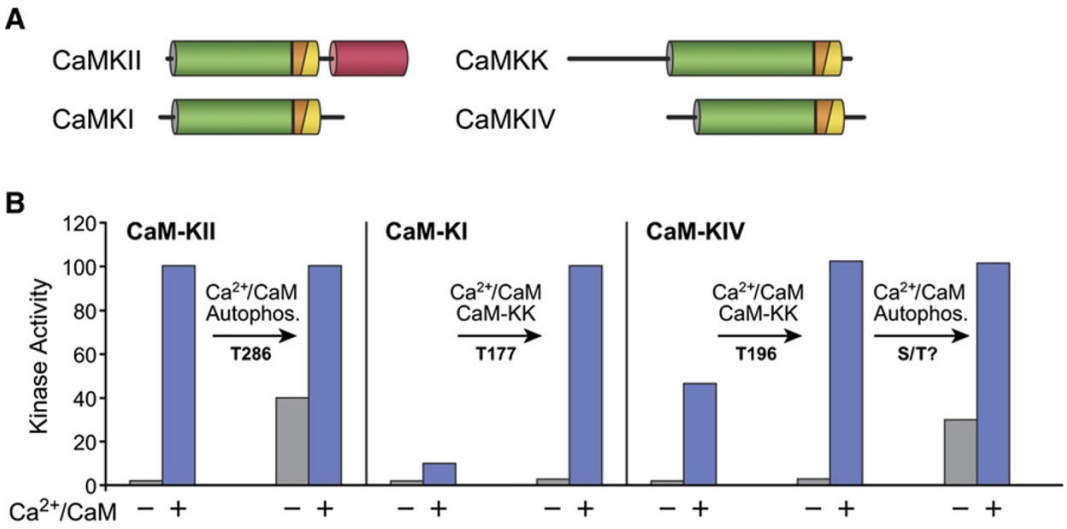

Figure 1. Schematic of CaM-Kinase Domains and Activation Properties

(A) CaM-kinase domains: catalytic domains, green; autoinhibitory domains, orange; Ca^{2+}/CaM-binding domains, yellow; association domain (CaMKII), red. (Reproduced by permission from Soderling and Stull, 2001).

(B) Effects of Ca^{2+}/CaM and autophosphorylation and/or phosphorylation on total kinase activity (+Ca^{2+}/CaM, blue) or autonomous activity (−Ca^{2+}/CaM, gray). The putative autophosphorylation site (S/T?) in CaMKIV is unknown. See text for details (Structure and Regulatory Mechanisms).

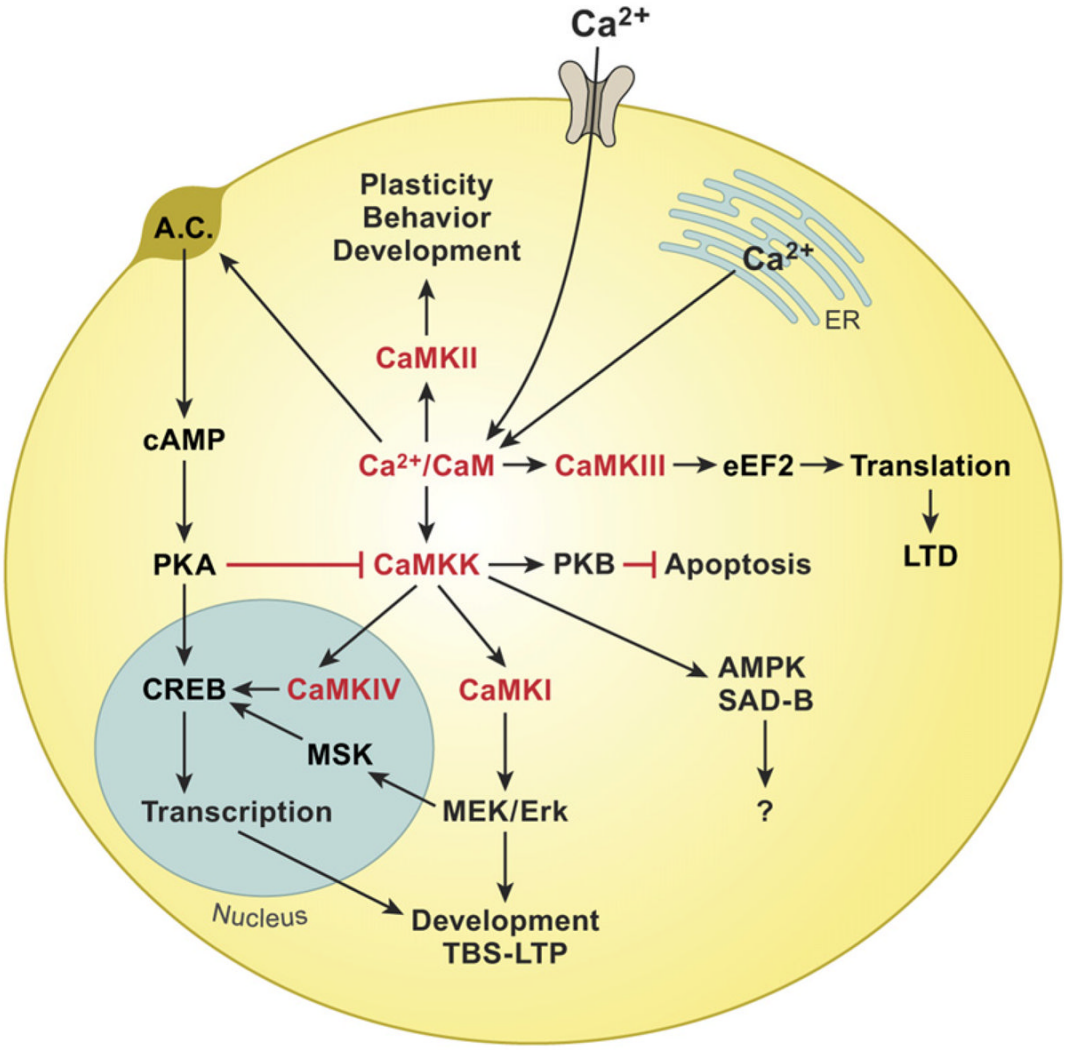

Figure 2. Cellular Signaling by CaMKs

Elevated intracellular Ca^{2+}/CaM bind to and activate CaMKII, CaMKK, CaMKI, and CaMKIV. CaMKII autophosphorylates to generate significant autonomous activity (see Figure 1). Primary targets of CaMKK are CaMKI (cytosolic) and CaMKIV (nuclear)—these require binding of Ca^{2+}/CaM to both CaMKK and CaMKI/CaMKIV (see Figure 1). Secondary substrates of CaMKK, which are phosphorylated at much slower rates than CaMKI and CaMKIV, are PKB/Akt and members of the AMP-kinase family, including SAD-B. Crosstalk between adenylyl cyclase (A.C.)/PKA to inhibit CaMKK and between CaMKI to activate MEK/Erk are illustrated. See text for details.

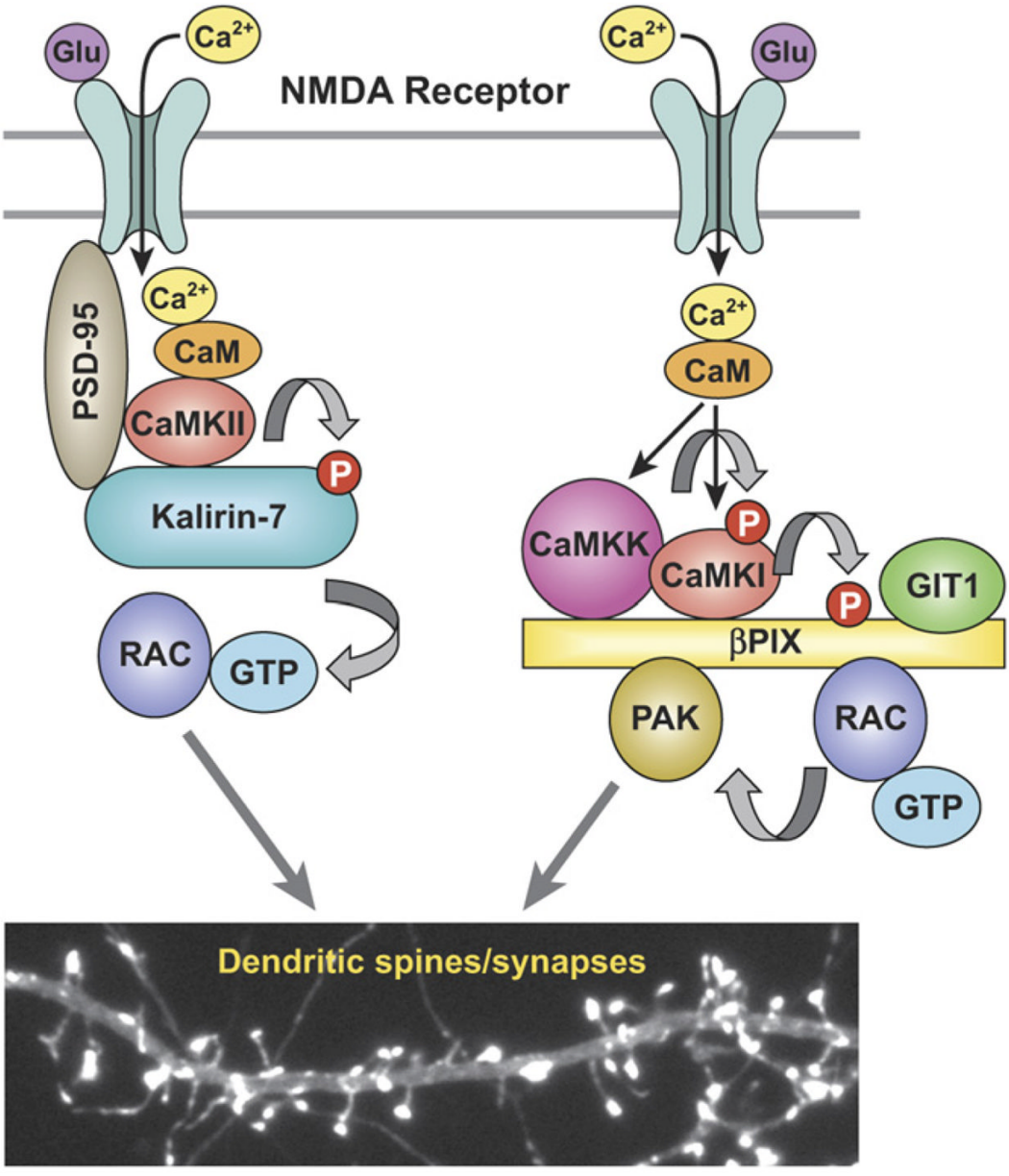

Figure 3. Regulation of Spine/Synapse Formation by CaMKs

Neuronal activity, via Ca^{2+} influx through the NMDAR, can activate both CaMKII and CaMKK/CaMKI. (Left side) CaMKII is complexed with PSD-95 and the Rac GEF kalarin-7, which it phosphorylates and activates to promote Pak-mediated actin formation and dendritic spines. (Right side) Similarly, CaMKK and CaMKIα are part of a multiprotein complex with the Rac GEF βPIX and GIT1, which localizes it in spines. CaMKI phosphorylates and activates βPIX to stimulate Rac- and Pak-mediated spine and synapse formation.

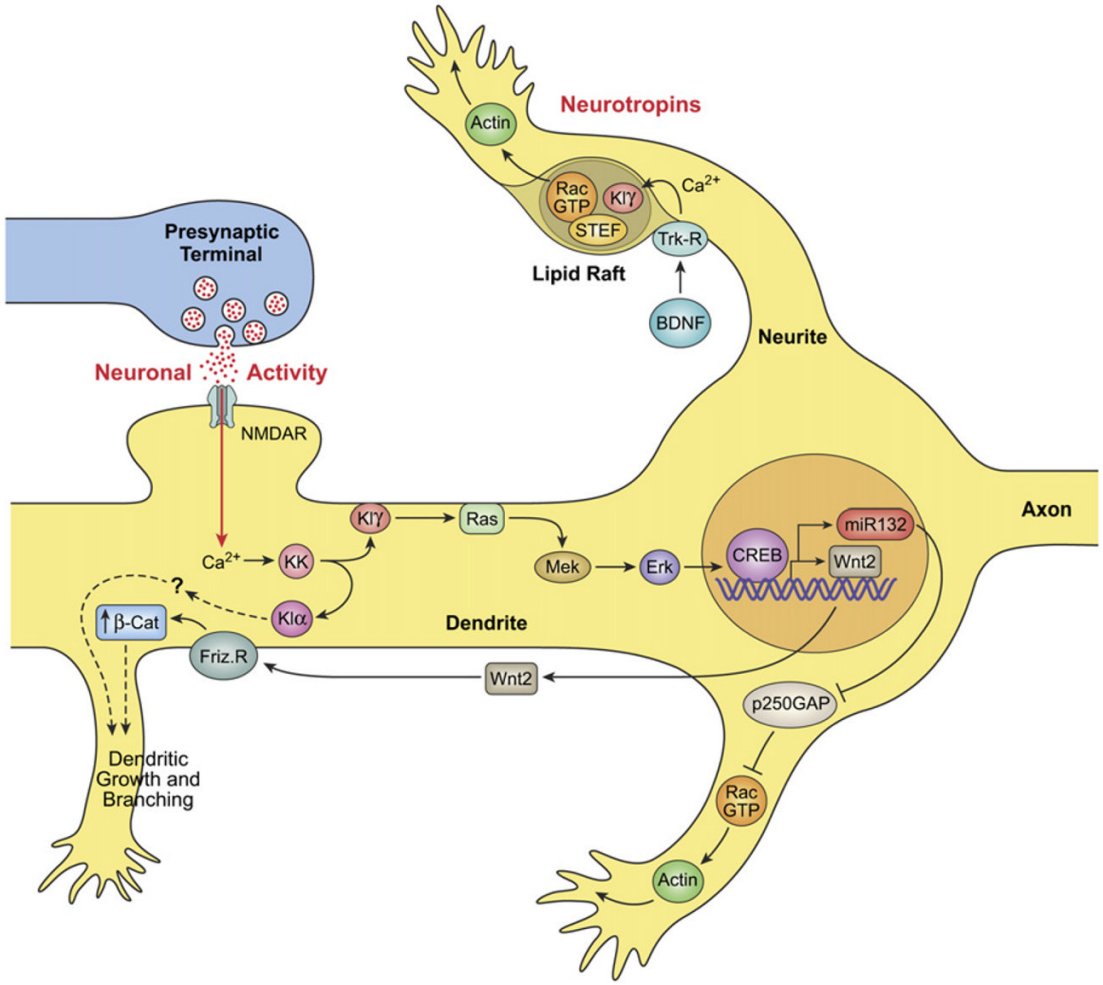

Figure 4. Regulation of Dendritic Development by CaMKγ

(Top right) Neurotropins. Initial outgrowth of neurites to form dendrites (total length ~100 μm) is regulated in part by spontaneous and/or neurotropin-mediated (e.g., BDNF) activation of lipid-modified CaMKIγ that is colocalized in a lipid raft with Rac and its GEF, STEF. This signaling pathway is postulated to enhance actin polymerization and thereby promote dendrite formation. Neuronal Activity. Continued dendritic arborization (total length >1000 μm) via neuronal activity requires Ca^{2+} influx through the NMDAR to activate CaMKK and both α and γ CaMKI. The CaMKIγ activates the MEK/Erk pathway to stimulate CREB-dependent synthesis of Wnt-2 and microRNA132 (miR132). Wnts are known to stimulate dendritic development via β-catenins, and miR132 suppresses translation of p250GAP, thereby stimulating Rac1 and dendritic outgrowth. Downstream targets of CaMKIα in this system remain to be identified—potential candidates are discussed in the text (Neuronal Development and Table 1). Modified figure used by permission (Ciani and Salinas, 2008).

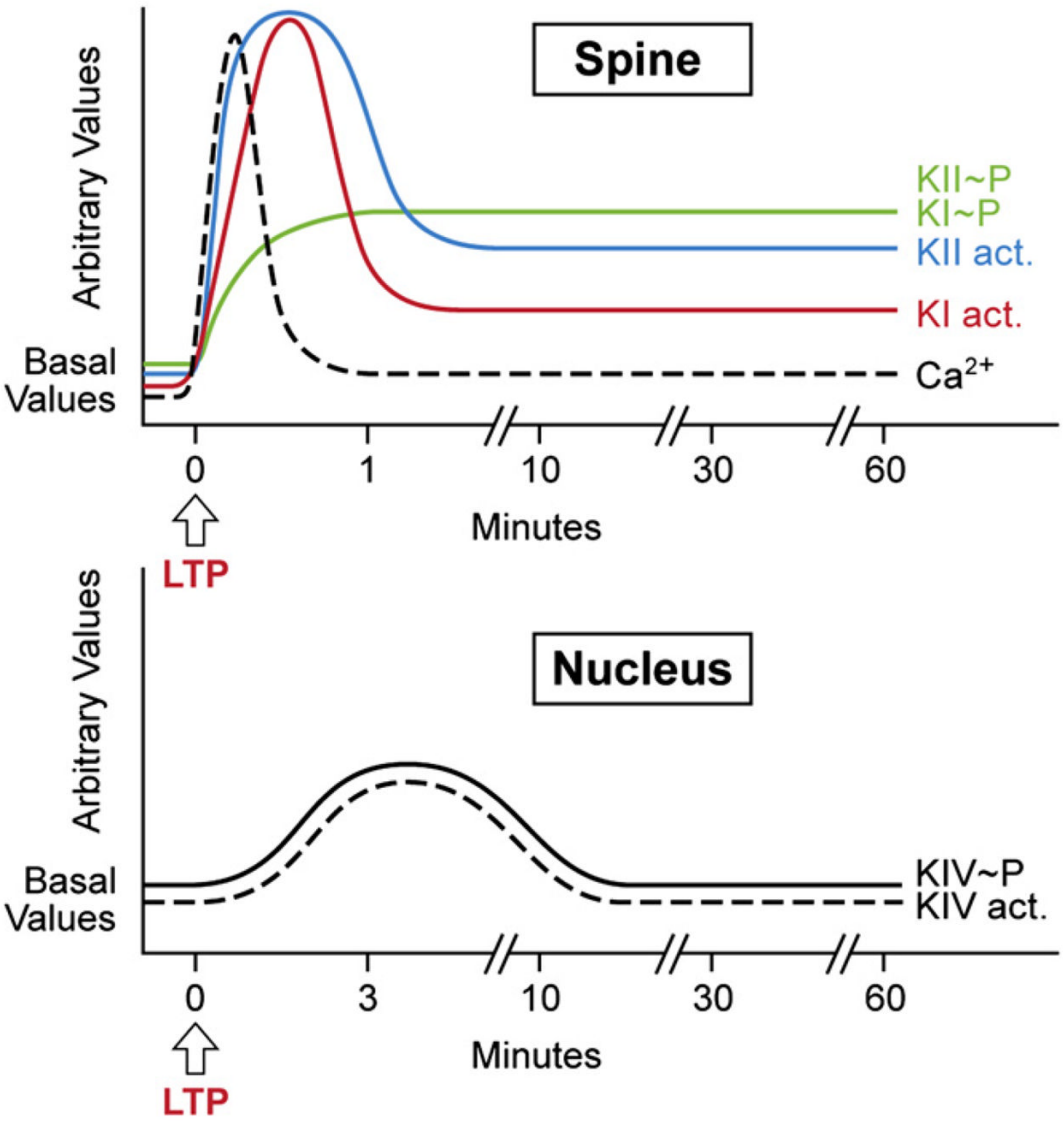

Figure 5. Schematic of CaMK Activations by Long-Term Potentiation

(Top) Hippocampal CA1 LTP induction elicits an NMDAR-mediated spike of $[Ca^{2+}]_I$ in dendritic spines. This elevated Ca^{2+}/CaM triggers rapid CaMKII activation (KII act) and intramolecular autophosphorylation (KII~P) to generate autonomous CaMKII activity that persists, even after slow dissociation of Ca^{2+}/CaM, for at least 1 hr. This autonomous CaMKII activity can phosphorylate numerous substrates in the postsynaptic density that may contribute to synaptic potentiation. The elevated spine Ca^{2+}/CaM also activates CaMKK to phosphorylate (KI~P) and activate CaMKI (KI act). Although CaMKI phosphorylation persists for at least 1 hr, its activity is more transient since autonomous activity is not generated (see Figure 1). However, even when $[Ca^{2+}]_I$ returns to basal values, CaMKI will remain somewhat activated by basal Ca^{2+}/CaM due to its activity via CaMKK phosphorylation. If the LTP induction protocol results in CaMKI-mediated transient incorporation of Ca^{2+}-permeable AMPARs, the $[Ca^{2+}]_I$ should remain somewhat elevated for ~20–25 min due to spontaneous activation of the AMPARs (data not shown). This source of $[Ca^{2+}]_I$ may uniquely activated Ca^{2+}-dependent proteins necessary for maintenance of the LTP. (Bottom) LTP induction also promotes a transient (3–10 min) phosphorylation (KIV~P) and activation (KIV act) of CaMKIV in the

nucleus that is thought to promote CREB-dependent gene transcription that may be necessary for maintenance of LTP. See section on Synaptic Plasticity for details.

Table 1

Substrates of CaMKI Identified In Vitro and/or In Situ

Protein	Phosphorylation Site	Regulatory Consequence of Phosphorylation	Predicted or Demonstrated Physiological Effect	Reference
βPix	Ser516	increased Rac GEF activity	enhance actin polymerization, spinogenesis	Saneyoshi et al., 2008
MARKS2/Par-1b	Thr294 Ser92	increased kinase activity	increased axonal outgrowth	Uboha et al., 2007
nNOS (neuronal nitric oxide synthase)	Ser741	inhibition of nNOS activity	unknown	Song et al., 2004
Numb/Numbl	Ser264/Ser 304 Numb/Numbl	recuitment of 14-3-3 and decreased binding to AP-2	endocytosis?	Tokumitsu et al., 2005, 2006
MRLC (Myosin II Regulatory Light Chain)	Ser19	increased Mg-ATPase activity of Myosin II	Actin dynamics	Suizu et al., 2002
CREB (cAMP response element-binding protein)	Ser133	transcriptional activation	transcriptional activation	Sheng et al., 1991
ATF1 (activating transcription factor)	Ser63	transcriptional activation	transcriptional activation	Sun et al., 1996
CFTR (cystic fibrosis transmembrane conductance regulator)	unknown	unknown	unknown	Picciotto et al., 1992
Translational initiation factor 4GII	Ser1156	unknown	unknown	Qin et al., 2003
HDAC-5 (histone deacetylase)	Ser259, Ser498	recuitment of 14-3-3 resulting in disruption of MEF2-HDAC complex and nuclear export of HDAC5	blocks MyoD-dependent conversion of fibroblast to muscle	McKinsey et al., 2000
HSP-25 (25 kDa heat shock protein)	Ser15 + Ser85(minor site)	unknown	unknown	Corcoran et al., 2003
p300 (transcriptional co-activator)	Ser89	suppress histone acetyltransferase activity	repress transcription	Corcoran et al., 2003; Yuan et al., 2002