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CaMKII: a central molecular organizer of synaptic plasticity, learning and memory

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Abstract | Calcium–calmodulin (CaM)-dependent protein kinase II (CaMKII) is the most abundant protein in excitatory synapses and is central to synaptic plasticity, learning and memory. It is activated by intracellular increases in calcium ion levels and triggers molecular processes necessary for synaptic plasticity. CaMKII phosphorylates numerous synaptic proteins, thereby regulating their structure and functions. This leads to molecular events crucial for synaptic plasticity, such as receptor trafficking, localization and activity; actin cytoskeletal dynamics; translation; and even transcription through synapse–nucleus shuttling. Several new tools affording increasingly greater spatiotemporal resolution have revealed the link between CaMKII activity and downstream signalling processes in dendritic spines during synaptic and behavioural plasticity. These technologies have provided insights into the function of CaMKII in learning and memory.

Protomers
Individual protein molecules in a protein complex.

Calcium–calmodulin (CaM)-dependent protein kinase II (CaMKII) has remarkable properties and functions. When activated, CaMKII undergoes autophosphorylation, thus rendering it active independent of the calcium–CaM complex (hereafter abbreviated as Ca^{2+}–CaM), beyond the initial stimulation — endowing the kinase with a 'molecular memory'[1–3]. In the hippocampus, CaMKII accounts for about 1–2% of total protein by mass and about 10% of protein in the postsynaptic density (PSD)[4–7]. The PSD is a protein-dense meshwork that constitutes the postsynaptic sites of glutamatergic synapses, typically in dendritic spines, the small protrusions of dendrites of excitatory neurons.

In addition to its catalytic functions, CaMKII has essential structural functions, many of which depend on its autophosphorylation and calcium[8]. Thus, it has been proposed that CaMKII acts to a large degree as an activity-dependent structural protein[2,8,9]. CaMKII has important roles in synaptic plasticity, including in long-term potentiation (LTP) and spine structural plasticity, and in the formation of many types of memory, providing a key argument for the hypothesis that LTP is part of physiological underpinning of memory[1,2]. CaMKII is also involved in long-term depression (LTD)[10] (reviewed elsewhere[3]). Impaired CaMKII function is associated with intellectual disabilities, developmental delay and seizure activity, further supporting its crucial roles in synaptic plasticity[11–14].

The past decade has seen important progress in understanding the mechanisms underlying CaMKII signalling in synaptic plasticity, in part because new tools have enabled CaMKII signalling to be imaged and manipulated at the level of single synapses and even down to the nanometre scale. In addition, recent structural and biochemical studies have provided fresh insights into the fine mechanistic details of how CaMKII works. The goal of this Review is to integrate recent findings that shed new light on postsynaptic CaMKII regulation and function with earlier insights. Important new tools and concepts include the development of photoactivatable constructs of CaMKII and one of its inhibitors, the realization of the importance of liquid–liquid phase separation (LLPS) for postsynaptic CaMKII functions and the recognition of a continued binding groove that is shared by substrates, as well as CaMKII-associated proteins (CaMKAPs) and some CaMKII inhibitors.

Structure and activity

CaMKII possesses several unique features, including its ability to be acutely activated by Ca^{2+}–CaM and to be more enduringly activated by autophosphorylation, as well as its multimeric structure. This section introduces the structure and mechanisms of activation of CaMKII.

Structure and subunits of CaMKII. CaMKII protomers consist of a catalytic domain with a bilobed structure (as is typical for kinases), an autoinhibitory or regulatory segment, a linker of various lengths and an association domain, which mediates the formation of dodecameric

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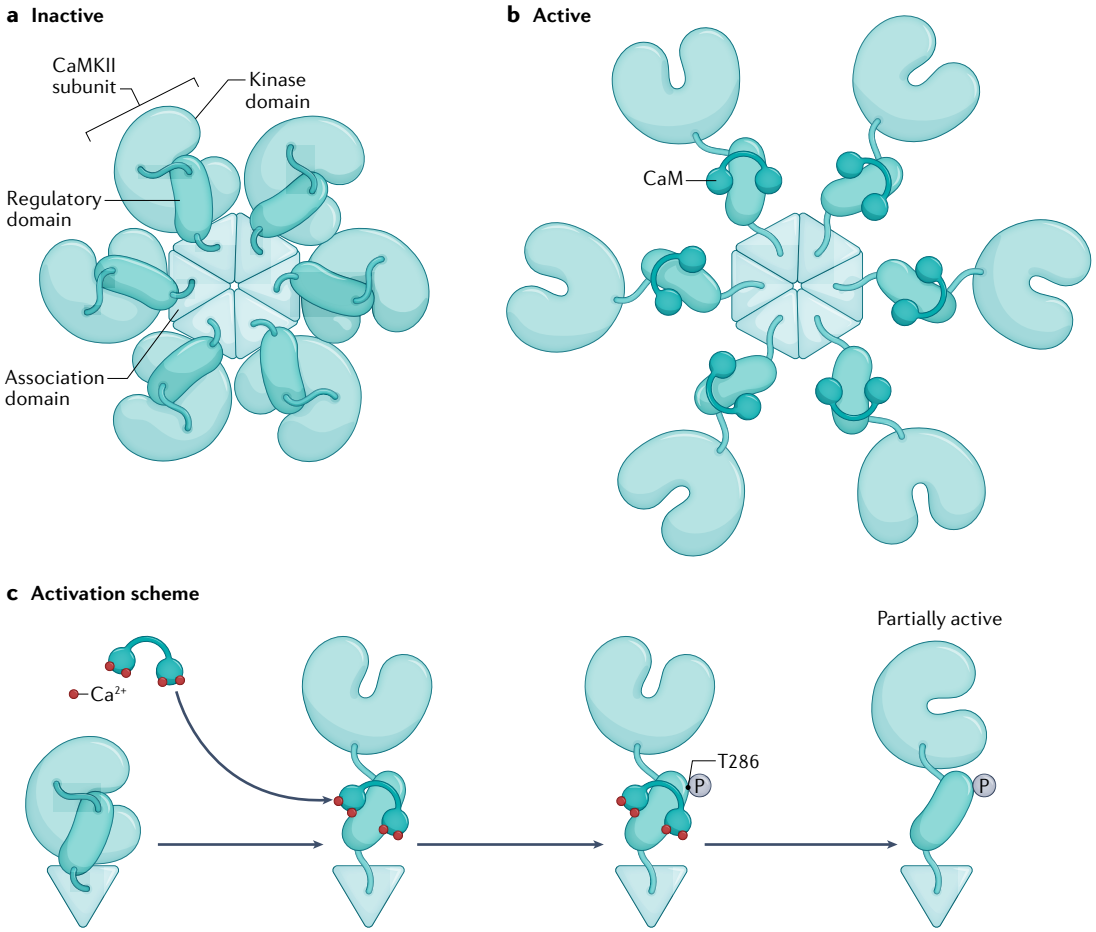


Fig. 1 | **Structure of CaMKII and its activation scheme. a** | Structure of calcium–calmodulin (CaM)-dependent protein kinase II (CaMKII). The holoenzyme consists of 12 subunits, each of which acts as a kinase. Each subunit includes an association domain, a regulatory domain and a kinase domain. For simplicity, CaMKII is drawn as a hexamer, but actually it is a dodecamer consisting of two hexameric rings stacked on top of each other, with both rotational and planar symmetry. **b** | Upon activation, each subunit changes its conformation from the closed form to the open form. **c** | The conformational change to CaMKII upon calcium–CaM binding and subsequent phosphorylation of T286.

holoenzymes[15,16] (FIG. 1). The CaMKIIα, CaMKIIβ, CAMKIIγ and CAMKIIδ subunits are, respectively, encoded by four different genes — *CAMK2A*, *CAMK2B*, *CAMK2G* and *CAMK2D*. The subunits can copolymerize to form either homomeric or heteromeric holoenzymes. CaMKIIα is expressed in excitatory neurons in the hippocampus and cortex and in some inhibitory neurons such as Purkinje cells in the cerebellum and medium spiny neurons in the striatum, whereas other subunits are expressed more widely among brain regions and neuronal cell types[17]. CaMKIIα, CaMKIIβ, and CaMKIIγ have roles in synaptic plasticity[18–21]. CaMKIIα and CaMKIIβ are two of the most abundant PSD proteins in the hippocampus, making up approximately 10% of total protein in the PSD (about 960 monomers per 0.1 μm^2 of PSD), which is more than the prototypical postsynaptic scaffold protein PSD95 (about 250 monomers per 0.1 μm^2 of PSD) in both number and mass[4,7].

Holoenzymes
Fully functional enzymes with all components present.

Activation and autophosphorylation scheme of CaMKII. Each CaMKII subunit has three critical autophosphorylation sites on its regulatory domain[15] — in CaMKIIα, these are at T286, T305 and T306. In the inactive form of CaMKII, access to the catalytic domain is blocked by the autoinhibitory pseudosubstrate segment of the protein[15,16,22] (FIG. 1). Binding of Ca^{2+}–CaM to the adjacent regulatory segment removes the autoinhibitory segment from the catalytic site. The simultaneous binding of Ca^{2+}–CaM to two neighbouring subunits triggers CaMKII autophosphorylation at T286, which lies in the autoinhibitory domain[23], bestowing kinase activity that persists after Ca^{2+}–CaM dissociates from CaMKII (REFS.[24,25]) (FIG. 1). This so-called autonomous CaMKII activity has a crucial role in translating transient Ca^{2+} spikes into lasting CaMKII activation[26]. T286 phosphorylation also increases the affinity of CaMKII for Ca^{2+}–CaM by between 10-fold and 1,000-fold[27,28]. Thus, T286 autophosphorylation of multiple subunits in a holoenzyme provides a nonlinear translation of Ca^{2+} spike frequency and amplitude into kinase activity, in vitro and in neurons[27,29]. By contrast, the other autophosphorylation sites, T305 and T306, are located in the Ca^{2+}–CaM-binding site of the regulatory segment, and their phosphorylation prevents Ca^{2+}–CaM binding and kinase activation[15,30,31] (FIG. 1).

Phosphatases
Enzymes that remove phosphate moieties from phosphorylated proteins, lipids or other substrates.

Bistability
A property whereby a system can have two stable equilibrium states.

Bulk phase
Bulk phase signalling refers to signalling mediated by a population of CaMKII that is generally not anchored to CaMKAP. This population senses global calcium ions and transmits signals to diffusing substrates, as opposed to local signaling via CaMKAP–CaMKII complexes, which occur in nanodomains.

Glutamate uncaging
A microscopic technique that uses photons to release glutamate from light-sensitive precursors, often used to induce long-term potentiation in spines.

CaMKII may exist in distinct populations in dendritic spines. CaMKII had been thought to provide a molecular memory owing to its autophosphorylation at T286 and resultant constitutive activation persisting after the decline in intracellular Ca^{2+} concentration[26]. When the T286 residues of two adjacent subunits are phosphorylated, rephosphorylation after phosphatases remove one of the phosphates can be fast[28]. This mechanism potentially causes bistability in the CaMKII autophosphorylation status, and some theories have suggested that when CaMKII has become autophosphorylated, its phosphorylation can be maintained for a long time — even years[32,33]. At the same time, imaging of changes in the conformation of CaMKII as a proxy for its activity in dendritic spines has suggested that the activity of CaMKII, at least in the bulk phase, persists for only about a minute[29,34,35]. In addition, most pharmacological inhibitors of CaMKII block LTP when they are introduced at relatively low concentrations before, but not after, LTP induction[25,36,37]. These studies suggest that CaMKII activity lasts for only a short time compared with the timescale of memory (FIG. 2).

By contrast, biochemical studies from tissue lysates suggest that CaMKII is partially active under basal conditions, and that its activity persistently increases in response to LTP induction[38,39]. Some CaMKII inhibitors at high concentrations can reduce basal transmission and impair LTP maintenance, supporting the presence of active CaMKII under basal conditions[40,41]. The CaMKII pools in spines that mediate these effects might be small and functionally defined by prolonged interactions of CaMKII with its various binding partners that enable them to contribute to the maintenance of basal transmission and long-term memory[42–44]. For example, CaMKII associates with the NMDA receptor (NMDAR) subunit GluN2B for an extended period, potentially having structural and/or catalytic roles in the maintenance of basal transmission and synaptic memory[1–3,42,43,45]. Given there might be about 100-fold more CaMKII molecules in spines than GluN2B, and the high binding affinity of these molecules for each other (nanomolar)[5,46], the pool of CaMKII bound with GluN2B is probably not effectively detected by imaging and may not be readily accessible to low concentrations of competitive inhibitors[40,41,44]. In the following sections, we separately discuss bulk-phase CaMKII, which is readily accessible by imaging and low concentrations of competitive inhibitors, and the local pools of CaMKII that interact with other molecules (FIG. 2).

Bulk-phase CaMKII in dendritic spines

The recent development of Förster resonance energy transfer (FRET)-based CaMKII sensors and two-photon FRET and fluorescence lifetime imaging microscopy techniques has enabled the activity of CaMKII in dendritic spines to be directly monitored during LTP and associated spine growth[29,34,35,47] (FIG. 3). By combination of these techniques with two-photon glutamate uncaging[48], which enables spatially and temporally coordinated synaptic activation, important details of CaMKII functions in spines have been revealed.

Biosensors for CaMKII activity. The most commonly used CaMKII sensor is Camui, an engineered CaMKII molecule with fluorophores at its amino and carboxy termini (N and C termini)[47]. The distance between

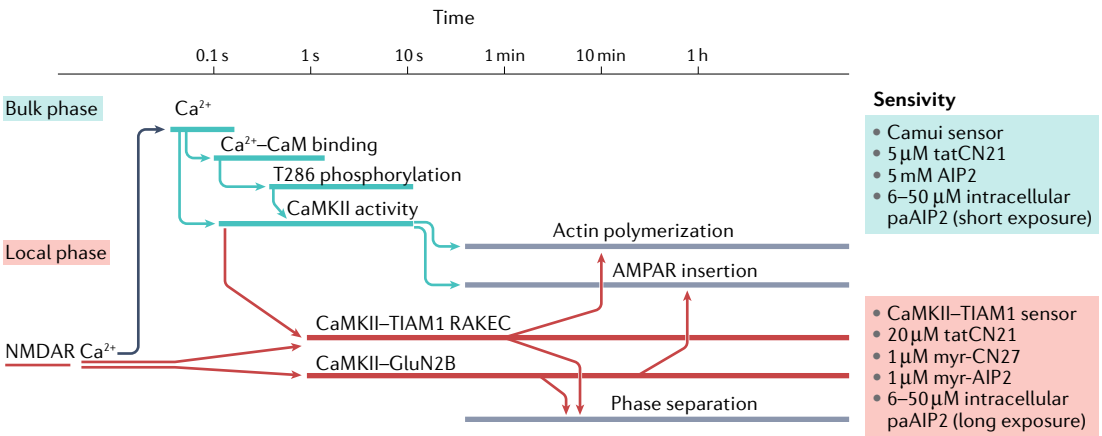


Fig. 2 | **The hypothetical timescale of CaMKII signalling in dendritic spines.** Transient increases in calcium levels (lasting 10–100 ms)[53] in a dendritic spine trigger cascades of biochemical events that lead to calcium–calmodulin (CaM)-dependent protein kinase II (CaMKII) signalling. CaMKII is activated by binding of the calcium–CaM complex (Ca^{2+}–CaM) (over the course of seconds[49,54]), and its activation is maintained by subsequent phosphorylation of T286. CaMKII activation occurs in the bulk phase (blue) and local nanodomain phase (red) in different time domains. The timescale of CaMKII activation in the bulk phase was measured by imaging and found to be transient (between seconds and 1 min (REFS.[34,35])). The exact timescale of local-phase CaMKII activity in dendritic spines is still unknown, but the interaction between CaMKII and CaMKII-associated proteins (CaMKAPs), such as the NMDA receptor (NMDAR) subunit GluN2B and the RHO guanine nucleotide exchange factor TIAM1, can persist for more than 30 min (REFS.[44,45]). The extracellular concentration of inhibitors affecting the bulk-phase and local-phase CaMKII-dependent processes are shown in boxes on the right side — with the exception of the light-inducible intracellular CaMKII inhibitor photoactivatable autocamtide inhibitory peptide 2 (paAIP2), whose intracellular concentration was 6–50 µM (REF.[37]). AMPAR, AMPA receptor; CN27, 27-amino acid sequence from the CaMKII-inhibitory protein CaMKIIN1; myr, myristoylated; RAKEC, reciprocally activating kinase–effector complex; tatCN21, tat peptide fused with a 21-amino acid sequence from CaMKIIN1.

Decay time constant
The time when something decaying in exponential fashion becomes 1/*e*, where *e* is Napier's constant.

the fluorophores changes when the CaMKII part of the sensor is activated and undergoes conformational changes, enabling FRET-based imaging of its activation (FIG. 1). In vitro assays suggest that this sensor reports Ca^{2+}–CaM binding as well as T286 autophosphorylation, with the FRET signal upon T286 autophosphorylation being about 60% of that seen with Ca^{2+}–CaM[34,47,49]. This difference is qualitatively consistent with biochemical assays that indicate that CaMKII is several times more active when Ca^{2+}–CaM is present than when it is T286-phosphorylated but Ca^{2+}–CaM is removed[50,51]. One limitation of Camui is that it measures the conformational change of overexpressed CaMKII as a proxy for CaMKII activity. A new sensor called 'FRESCA' (FRET-based sensor for CaMKII activity) measures the balance of kinase and phosphatase activity using a synthetic CaMKII substrate peptide attached to fluorescent proteins[52]. Use of FRESCA in the future should help to define the spatiotemporal dynamics of different CaMKII pools.

Baseline signalling. The percentage of CaMKII in the active or phosphorylated state in spines has not been measured. However, excitatory neurons exhibit low resting calcium ion concentrations (20–50 nM), and thus resting CaMKII activity is probably low[53,54]. Indeed, Camui-imaging studies show that wild type Camui signal under resting conditions is similar to that of the T286A mutant Camui (which cannot undergo T286 autophosphorylation), and the T305D–T306D mutant (which cannot bind to Ca^{2+}–CaM)[49]. Thus, under basal conditions, the effects of T286 phosphorylation and CaM binding on CaMKII activity seem to be below the detection limit of this method (about 10% of CaMKII activation during LTP)[34,35]. Sensors with higher signal-to-noise ratios should provide improved quantitative assessments of CaMKII activity under basal conditions.

Calcium level elevation and CaM binding. After calcium ions flow into a dendritic spine, they are extruded from the spine in 10–100 ms (REF.[53]). During this period, CaM binds to Ca^{2+}, and the resultant Ca^{2+}–CaM binds to CaMKII (REF.[54]). The association of CaM with CaMKII was imaged with a FRET-based sensor that consisted of enhanced green fluorescent protein (eGFP)–CaMKII and mCherry–CaM. The decay time constant without autophosphorylation was determined to be approximately 1 s (REF.[49]), consistent with computer simulations[54] (FIGS. 2 and 3), whereas T286 autophosphorylation prolonged this decay time to several seconds to minutes (see later).

Autophosphorylation of T286. When two adjacent CaMKII subunits bind Ca^{2+}–CaM, they undergo autophosphorylation at T286[1,23,35,55,56] (FIG. 1), prolonging their

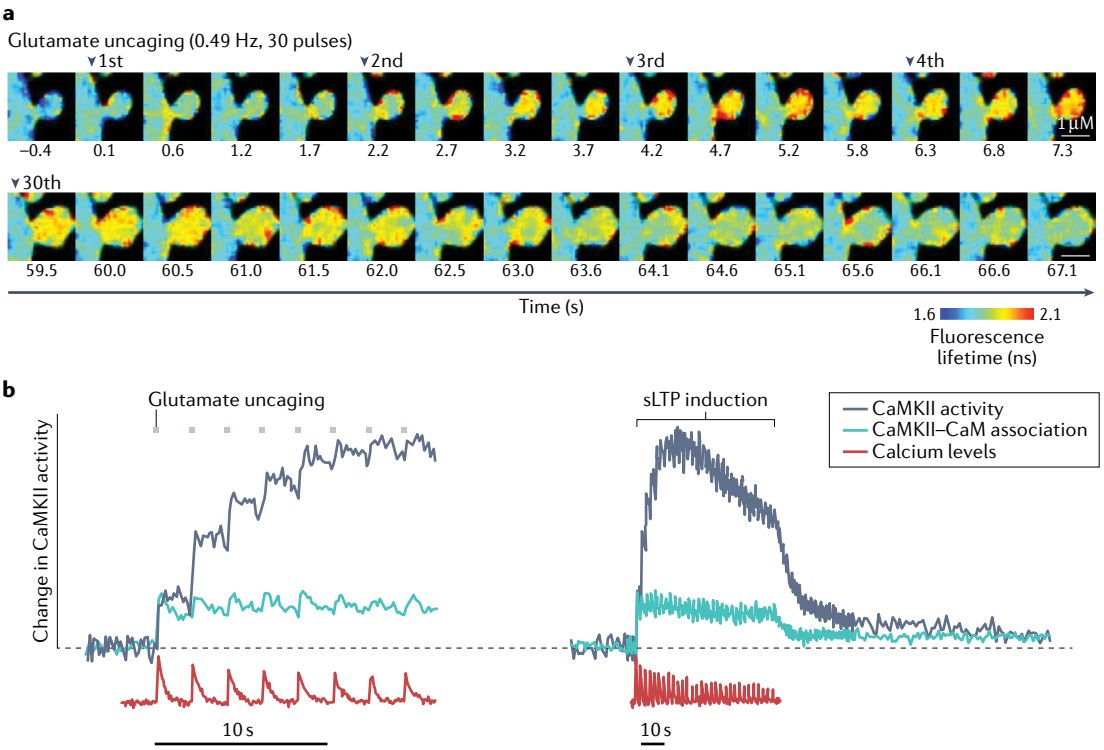


Fig. 3 | **Activation of CaMKII in single dendritic spines. a** | Activation of calcium–calmodulin (CaM)-dependent kinase II (CaMKII) imaged by two-photon fluorescence lifetime imaging microscopy and the Camui CaMKII sensor. CaMKII activity in single dendritic spines can be imaged with subsecond temporal resolution using the Camui sensor during spine structural long-term potentiation (LTP). **b** | CaMKII activation time course compared with CaM–CaMKII interaction and increases in calcium levels. CaMKII activity accumulates over the course of about 10 s, whereas the CaM–CaMKII interaction is saturated in about 1 s (REF.[49]). This difference is due to the autonomous, phosphorylation-dependent but Ca^{2+}–CaM-independent CaMKII activity. Part **a** is adapted with permission from REF.[35], Elsevier. Part **b** is adapted with permission from REF.[49], Springer Nature Limited.

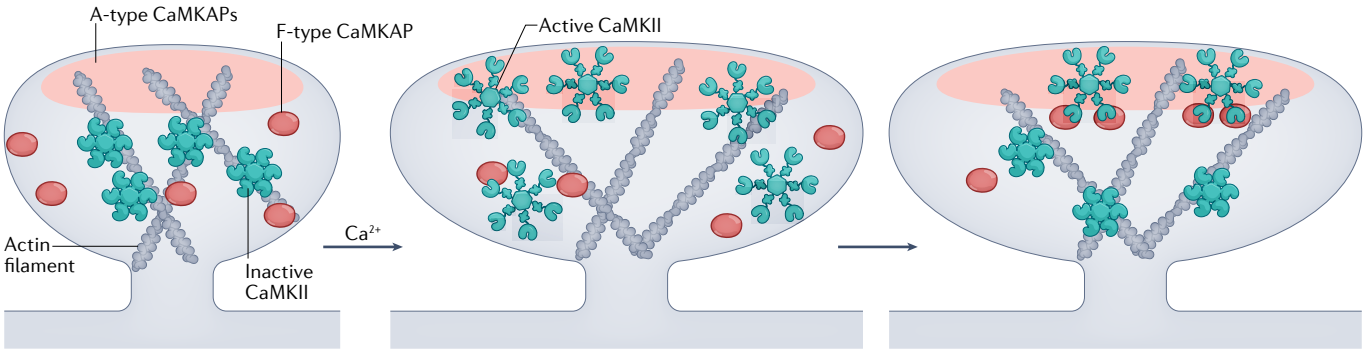


Fig. 4 | **Activity-dependent CaMKII association changes the localization of CaMKAPs during long-term potentiation.** Calcium–calmodulin (CaM)-dependent kinase II (CaMKII, shown in teal) is associated with various proteins in an activity-dependent manner. Increases in the level of calcium in spines can trigger changes in the localization of CaMKII and CaMKII-associated proteins (CaMKAPs) during synaptic plasticity. Anchoring-type (A-type) CaMKAPs, including F-actin (actin filaments shown in dark grey) and NMDA receptor subunit 2B (GluN2B), define the localization of CaMKII, whereas follower-type (F-type) CaMKAPs, including the RAC guanine nucleotide exchange factor TIAM1, activity-regulated cytoskeleton-associated protein (ARC) and proteins of the proteasome, are recruited by CaMKII.

binding to Ca^{2+}–CaM severalfold in spines[49], consistent with cell-free assays[27]. In addition, autophosphorylation at T286 enables CaMKII to integrate calcium signalling over about 6 s (REFS.[35,49]) (FIGS. 2 and 3). Given that there is no increase in CaMKII–CaM association beyond about 1 s after the decline in Ca^{2+} concentration, most of this integration over seconds is perhaps due to the autonomous activity of CaMKII, as overall CaMKII activation continues to increase over seconds to minutes during CaMKII–CaM association[35,49] (FIGS. 2 and 3). CaMKII deactivates with a time constant of about 1 min, which is slower than that for Ca^{2+}–CaM association (1 s) and the fast component of CaMKII activity decay (about 6 s)[35,49]. The biochemical mechanism of this slow decay is not clear, but it could be explained by an additional phosphorylation state or association with other proteins that protect CaMKII from phosphatase action[49]. Inhibitory phosphorylation at T305 and/or T306 seems to have only minor effects on the rate of decay[49].

T286 phosphorylation seems to prolong CaMKII activation by severalfold, enabling CaMKII to integrate short periods of calcium signalling (~100 ms). In other words, T286 phosphorylation regulates the time constant of the integrator function of CaMKII (FIG. 3). Because the decay time constant of T286A-mutant CaMKIIα is about 1 s, it is possible to induce LTP or spine enlargement in CaMKII T286A knock-in mice by stimulating spines at higher frequency (for example, with glutamate uncaging at 8 Hz in low extracellular Mg^{2+} conditions, or with 40-Hz electrical stimulation of depolarized postsynaptic neurons)[35]. Structural plasticity of dendritic spines can also be induced in CaMKII T286A knock-in mice by high-frequency stimulation, even when CaMKIIβ expression is also knocked down[35]. These findings suggest that CaMKII T286 phosphorylation is required to integrate the activity of CaMKII beyond the duration of Ca^{2+} concentration elevations but not to maintain LTP and structural plasticity.

Timescale of endogenous CaMKII signalling in dendritic spines. Imaging CaMKII activity provides the timescale of CaMKII activity in single dendritic spines. However, the timescales of the activity of overexpressed exogenous sensors may differ from those of endogenous CaMKII activity. Compounds that inhibit substrate phosphorylation by CaMKII generally inhibit LTP induction but do not reverse established LTP[25,36], suggesting that the timescale of endogenous CaMKII activity in spines is roughly consistent with imaging results[34,35]. The recent development of the genetically encoded photoinducible CaMKII inhibitor photoactivatable autocamtide inhibitory peptide 2 (paAIP2) has enabled the timescale of CaMKII signalling to be resolved[37]. Photoactivation of paAIP2 inhibits LTP and structural LTP (sLTP)[48] during their induction, but not when the photoinhibition of CaMKII is delayed by 1 min, suggesting that CaMKII activation is required for only about 1 min during induction[37], consistent with imaging results[34,35]. Thus, two methods — imaging and photoinhibition — have determined the timescale of CaMKII in the bulk phase to be in the range of seconds to about 1 min.

Local population action of CaMKII

Kinases are slow enzymes (typically carrying out 0.1–10 reactions per second); thus, tethering kinases near their substrates is a key strategy for achieving the necessary efficiency of the kinase action[57,58]. Carrying out 10 reactions per second[50], CaMKII is no exception. Many CaMKAPs bind to the substrate-binding region of CaMKII, affecting synaptic plasticity (FIG. 4). The high abundance of CaMKII (about 100 μM) at the postsynaptic site enables even a relatively weak interaction to cause a substantial degree of association. Given its dodecameric structure, CaMKII can crosslink various postsynaptic CaMKAPs, including GluN2B, densin 180 (also known as leucine-rich repeat-containing protein 7 (LRRC7)) and α-actinin[59]. Some interactions are induced by Ca^{2+}–CaM, whereas others (such as those with F-actin) are disrupted by it. Notably, for some proteins, including GluN2B and TIAM1, the Ca^{2+}–CaM-induced interaction can persist even after dissociation of Ca^{2+}–CaM[42,44,60]. Hence, similarly to T286 phosphorylation, such protein–protein interactions have been proposed to provide another mechanism for molecular memory during LTP[61].

Structural LTP
(sLTP). An activity-induced, lasting increase in spine size, mirroring electrically induced long-term potentiation (LTP).

Guanine nucleotide exchange factor
(GEF). A protein that activates small GTPases such as RAS and RHO by exchanging GDP on the small GTPases with GTP.

Condensates
Collection of molecules separated and condensed from the dilute phase through the process of liquid–liquid phase separation.

Apo state
The state in which an enzyme lacks one or more constituents required for its activity.

Ultrastructural distribution
Distribution of molecules at the nanometre scale, as determined by electron microscopy.

Exchange time
The time required for molecules in a compartment to be replaced with molecules in another compartment.

CaMKAPs can anchor CaMKII at specific nanodomains, such as the PSD or cytoskeleton, or can be follower or client proteins that are recruited to these sites through CaMKII binding. In this Review, we use the term 'anchoring (A)-type CaMKAPs' for the former and 'follower (F)-type CaMKAPs' for the latter. A-type CaMKAPs include GluN2B[42], L-, T- and P/Q-type voltage-gated calcium channels[62–65], F-actin[66–68] and microtubule proteins[69]. F-type CaMKAPs include the RAC guanine nucleotide exchange factor (GEF) TIAM1 (REF.[44]), activity-regulated cytoskeleton-associated protein (ARC)[70] and proteasomal proteins[71,72], and are important for producing local nanodomain signalling[20,34,63]. Densin 180 and α-actinin can act as F-type or A-type CaMKAPs[2,73] (FIG. 4).

A unique CaMKAP is the L-type calcium channel $Ca_V1.2$. CaMKIIβ interacts with $Ca_V1.2$ and locally phosphorylates CaMKIIγ, facilitating the binding of Ca^{2+}–CaM to CaMKIIγ. Upon arrival of the complex at the nucleus, Ca^{2+}–CaM is released from the complex. Thus, through these reactions, CaMKIIγ shuttles Ca^{2+}–CaM into the nucleus, where Ca^{2+}–CaM stimulates cAMP response element-binding protein (CREB) phosphorylation via CaMK kinase (CaMKK) and CaMK type IV (CaMKIV)[20].

CaMKAPs and LLPS of CaMKII. The excess of CaMKII compared with GluN2B leaves a large number of kinase subunits that are available for binding with other proteins. Indeed, the number of identified CaMKAPs (A-type and F-type) is increasing, and currently includes TIAM1, densin 180, the L-type Ca^{2+} channel $Ca_V1.2$ and the small GTPase REM2 (REFS.[42,44,73–75]). Comparison of the amino acid sequence in these CaMKAPs that bind CaMKII in recent crystallographic analysis[46,76,77] reveals a consensus sequence of (R/K)-X_n-L-X-(R/K)-Q-X-(S/T)-φ-D (where *n* is 2 or 3, and φ symbolizes a hydrophobic amino acid; BOX 1). Notably, this overlaps with consensus phosphorylation site RXX(S/T), although not all of the binding partners of CaMKII are necessarily substrates, as some feature alanine instead of serine/threonine. Because there are more than 400 known CaMKII consensus phosphorylation sites (BOX 1 Figure part c), it is likely that there are more CaMKAPs that remain to be identified.

LLPS is emerging as a driving force for the formation of localized nanodomains that include CaMKII as well as AMPA receptors (AMPARs) and NMDARs. Weak and multimeric interactions between CaMKII and various CaMKAPs can produce condensates[78,79]. Also, CaMKII can change its binding partner depending on the activation status[79]. We postulate that the LLPS of CaMKII has two key roles in synaptic plasticity. First, through interactions with A-type CaMKAPs through the substrate-binding site, CaMKII is anchored and forms a condensate beneath the postsynaptic membrane in a calcium-dependent manner, trapping F-type CaMKAPs in the condensate through interactions via the same substrate-binding site. In this way, CaMKII may serve as a 'sponge' to sequester various postsynaptic proteins, thereby triggering Ca^{2+}-dependent trafficking of various proteins[61].

Remarkably, when CaMKII undergoes LLPS with AMPARs and NMDARs, the overall status of the droplet changes dramatically upon binding of Ca^{2+}–CaM to CaMKII, separating NMDAR-rich and AMPAR-rich subdomains within the protein condensates that form during LLPS (BOX 2). Furthermore, CaMKII can undergo LLPS with the synaptic protein SHANK3 (SH3 and multiple ankyrin repeat domains protein 3) without a need for Ca^{2+}–CaM or any of the aforementioned proteins[79]. Therefore, CaMKII can form several different condensates in spines depending on its activation status.

Interestingly, in its inactive apo state CaMKII binds to the N-terminal region of SHANK3 (REF.[79]), but when activated by Ca^{2+}–CaM, it binds instead to a central polybasic motif (Arg949-Arg950-Lys951)[80]. One possible interpretation of these data is that calcium-ion influx can induce a conformational change of the pre-existing CaMKII–SHANK3 complex, driving additional protein interactions and restructuring of the nanoscale environment of SHANK3. Phosphorylation of SHANK3 by CaMKII may play a part here[81]. These changes could occur at the sites of pre-existing CaMKII–SHANK3 complexes or could induce a redistribution of CaMKII from the spine interior to the PSD[82–84].

Activity-dependent localization changes of CaMKII. Increasing calcium levels globally — through bath application of glutamate, NMDA or KCl — induces activity-dependent binding of endogenous CaMKII to NMDARs[42,85] and redistribution of CaMKII from the dendritic shaft to spines[86,87]. This redistribution depends on the activity-driven binding of CaMKII to GluN2B[42,88] but little on T286 phosphorylation[42,89]. At the nanometre scale, redistribution also seems to occur from the spine interior to the PSD. Electron micrography has shown that, under basal conditions, the CaMKII distribution peaks about 40 nm from the PSD. By contrast, spines stimulated with high-concentration potassium solution display more CaMKII near the PSD, whereas those inactivated by chelating extracellular calcium exhibit more cytosolic CaMKII distribution[83,90].

A super-resolution microscopy technique called 'single-particle tracking photoactivated localization microscopy' enables the ultrastructural distribution of CaMKII in dissociated live neurons to be measured without fixation artefacts. A study using this technique[91] found that CaMKII is distributed both at the PSD and away from the PSD, and that NMDAR activation increases the pool of CaMKII at the PSD, consistent with electron microscopy studies[83]. Furthermore, mobility analyses suggest that CaMKII is immobilized in spines both at and away from the PSD, probably through interaction with PSD-resident proteins and F-actin, respectively[92].

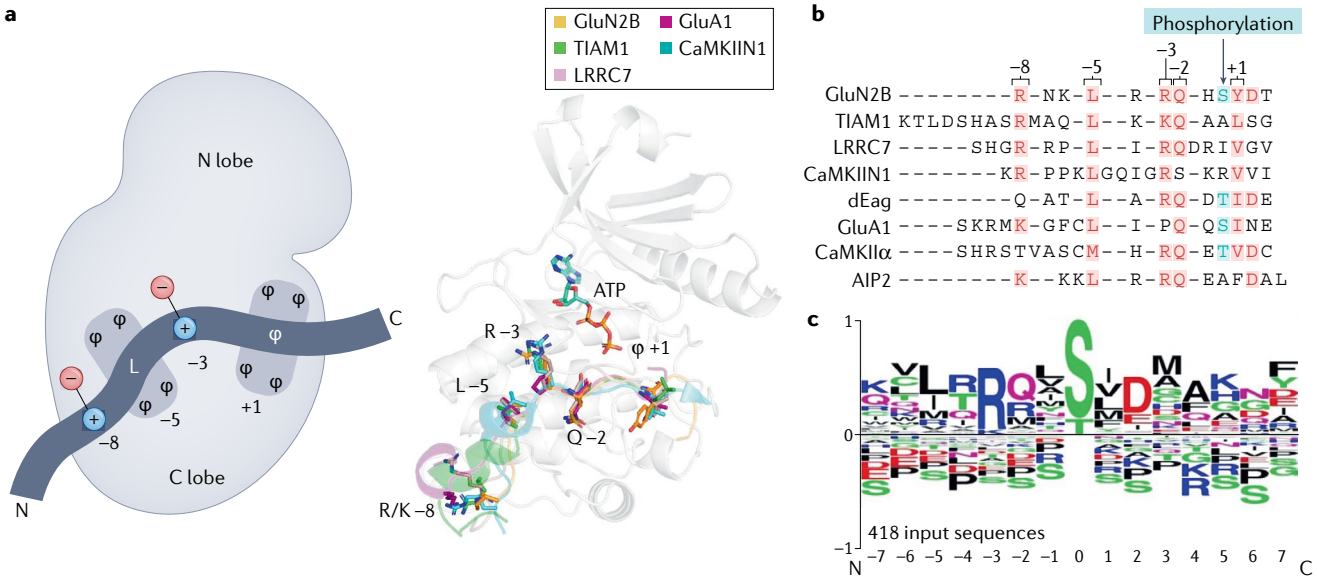
The diffusion constants measured by single-particle tracking broadly range from 10^{-3} to 10^{-1} μm² per second and define multiple populations of CaMKIIα (REF.[91]). The fast mobile pool (about 10^{-1} μm² per second) is consistent with the exchange time of the highly mobile pool of CaMKII as measured by fluorescence recovery after photobleaching (about 1 min)[34,62,93,94]. This pool

Box 1 | **Structural basis of activity-dependent binding of CaMKII and binding partners**

Early studies proposed that calcium–calmodulin (CaM)-dependent kinase II (CaMKII) has two discrete binding sites: the substrate-binding site (S-site) and the T-site, which is where the region of the autoinhibitory domain around T286 docks[42,89]. Some CaMKII-associated proteins (CaMKAPs), including GluN2B, can bind both to the T-site and to the S-site. After GluN2B initially binds to the S-site and is phosphorylated, it dissociates from the S-site and binds to the T-site[89]. This model explained the biochemical assays that have suggested that GluN2B binding to the T-site can lock CaMKII into an active conformation[89], with the S-site remaining open for substrate binding. However, recent studies resolving X-ray crystallographic structures of the CaMKII kinase domain bound to GluN2B and other peptides may force us to consider an alternative model.

The resolved structures include those of CaMKIINtide, which is derived from the CaMKII-inhibitory protein CaMKIIN1 (Protein Data Bank (PDB) identifier 3KL8)[77], the *Drosophila* potassium voltage-gated channel protein dEag (PDB identifier 5H9B)[76], GluN2B (which binds to the T-site and the S-site of CaMKII; PDB identifier 7UJP), TIAM1 (which binds to the T-site of CaMKII; PDB identifier 7UIR), densin 180 (also known as leucine-rich repeat-containing protein 7 (LRRC7), which binds to the S-site of CaMKII; PDB identifier 6X5G) and GluA1 (which contains a S831 phosphorylation site; PDB identifier 6X5Q)[46]. An alignment of these structures shows that they all bind CaMKII essentially in the same manner (see the figure, parts **a**,**b**, and REF.[46]). When the amino acid sequence of CaMKII-binding proteins is aligned to 0 at a phosphorylation site in CaMKII, the critical residues that these proteins tend to share are R/K at –8, L at –5, R at –3, Q at –2 and φ at +1. Upon alignment of known CaMKII phosphorylation sites of its substrates from the database PhosphoSitePlus, the consensus sequence of L-X-R-Q/R-X-S/T-φ-D emerges (see the figure, part **c**), consistent with key residues of T-site-binding peptide from crystallographic data.

The crystallographic data show that there is one continuous binding site, rather than discrete S-sites and T-sites, which also explains why ATP binding to the catalytic S-site enhances binding to GluN2B[160]. It remains unresolved how GluN2B and TIAM1 lock CaMKII into an active conformation. The crystal structures[46,76,77] also do not explain why binding some proteins, such as CaMKIIN1 and LRRC7, inhibits the kinase activity of CaMKII, whereas GluN2B and TIAM1 binding does not. Because these crystallographic data were obtained with a truncated monomeric form of CaMKII (residues 7–274), it is conceivable that the binding of some CaMKII subunits to certain (but not all) CaMKAPs induces an overall conformation of the CaMKII holoenzyme that enables CaMKII subunits that are not engaged in CaMKAP binding to become active independently of calcium–CaM binding or T286 phosphorylation. AIP2, autocamtide inhibitory peptide 2; C lobe, carboxy-terminal lobe; N lobe, amino-terminal lobe. Figure adapted with permission from REF.[46], Elsevier.



may represent cytosolic, unbound CaMKII molecules. However, overall the diffusion speed of CaMKII is slower than that expected from the hydrodynamic radius (about 10 μm² per second)[95,96]. The low-mobility fraction of CaMKII probably includes the populations bound to immobilized F-actin or PSD proteins as a result of LLPS[67,91,92,97–99].

Overall, these nanometre-scale studies suggest that CaMKII has at least three modes of distribution — one cytosolic, one at the PSD and the other near F-actin — and that the balance in distribution of the CaMKII pool between these loci shifts towards the PSD in an NMDAR-dependent manner[86]. Further molecular studies have suggested that CaMKII localization at the PSD is mediated at least to a substantial degree by the interaction between GluN2B and CaMKII (REFS.[88,89]), whereas CaMKII localization at the cytoskeleton is mediated through a CaMKIIβ–F-actin interaction[66,92,100]. In the following sections, we discuss specific interactions in detail.

Hydrodynamic radius
Radius of a molecule or protein with its full hydration shell, formed by water molecules.

Cytoskeleton. The CaMKIIβ subunit interacts with the actin cytoskeleton through its unique linker sequence and probably through several other sites[67,97–99]. Because a typical neuronal CaMKII holoenzyme can include multiple CaMKIIβ subunits, this interaction bundles and stabilizes actin filaments in spines[66,67] and probably contributes to the localization of CaMKII inside spines[19,101]. Ca^{2+}–CaM binding to the regulatory domain of CaMKII as well as the autophosphorylation at multiple serines or threonines in the F-actin-binding region of CaMKIIβ reduces the affinity for F-actin[92]. Therefore, CaMKIIβ is released from F-actin upon calcium-ion influx into spines, thereby creating a time window in which F-actin can be modified by actin-regulatory proteins[66,92,100]. Thus, CaMKIIβ activation gate sLTP and CaMKII–F-actin

PDZ domain
A protein domain that includes a shallow groove that binds the carboxy termini of defined proteins that typically end with Ser/Thr-X-Val, with X being any of the 20 protein-forming amino acids.

binding could provide a pool of CaMKII inside spines that can relocate moderately quickly during early phases of LTP. Consistent with this, mice expressing a variant of CaMKII that is constitutively phosphorylated at these F-actin-binding sites show impairments in structural plasticity and learning[60]. In addition, CaMKII can interact with microtubules in an activity-dependent but transient manner[69]. The interaction of CaMKIIα with α-actinin may also contribute to its interaction with F-actin[2,73].

GluN2B subunit of NMDARs. The interaction between GluN2B and CaMKII is important for LTP[2,40,43,88,102]. This interaction requires CaMKII to be in an open conformation, induced by either Ca^{2+}–CaM binding or T286 phosphorylation[42]. Thus, this interaction is driven by Ca^{2+} influx and consequent Ca^{2+}–CaM binding and CaMKII activation. During LTP, it mediates recruitment of CaMKII to the PSD, where NMDARs reside[88]. Hippocampal LTP is attenuated in knock-in mice expressing a variant of the NMDAR subunit GluN2B with crucial interaction sites mutated (L1298A and R1300Q)[88] or when mutant GluN2B (R1300Q and S1303D) is overexpressed in organotypic slices[102]. Upon calcium influx, as occurring during LTP, GluN2B binding strategically localizes CaMKII at the source of calcium influx and near AMPARs, the ultimate effectors in synaptic transmission and LTP. Indeed, in GluN2B L1298A–R1300Q knock-in mice, the activity-driven phosphorylation of the AMPAR subunit GluA1 on S831 by CaMKII is abolished[88].

As stated earlier, CaMKII outnumbers GluN2B by a large margin in PSDs. How, then, can binding of CaMKII to GluN2B be so central to recruitment of CaMKII to the PSD and to LTP? Possible explanations are that GluN2B-bound CaMKII can recruit additional CaMKII holoenzymes to the PDZ domain through direct

Box 2 | Phase separation and CaMKII

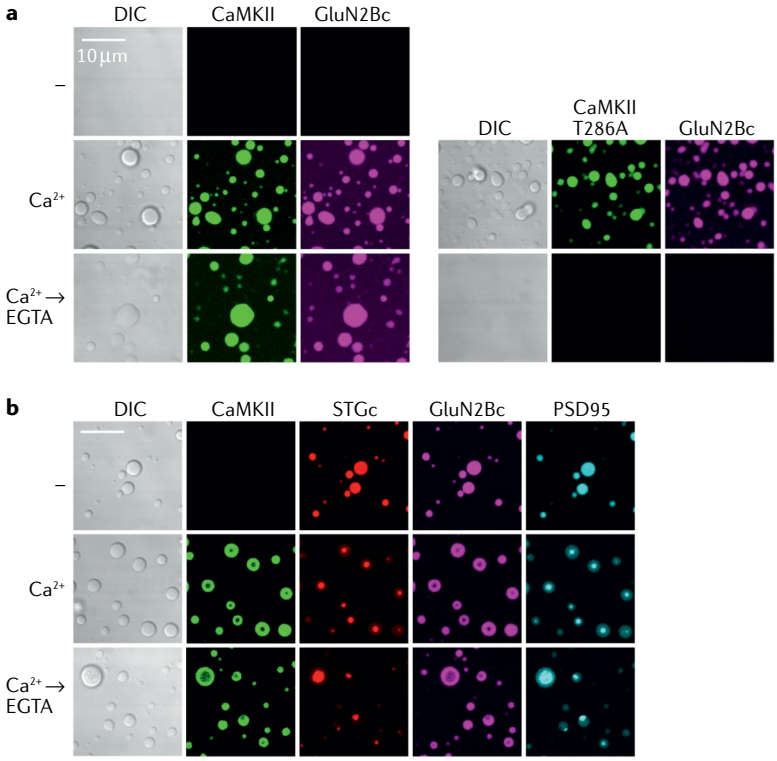
Liquid–liquid phase separation (LLPS) is an emerging concept in cell biology. Solutions containing biological macromolecules, such as proteins and nucleic acids, spontaneously separate into condensed and diluted phases[184,185]. The condensation of a select set of molecules in a small volume leads to the formation of membraneless compartments in the cell. Those molecules retain their native conformation and activity, and undergo dynamic exchange between diluted and condensed phases. The condensate retains properties of a liquid, distinct from protein aggregates, which are typically solid and can be irreversible.

LLPS often occurs among molecules with weak multivalent interactions[184,185]. Indeed, calcium–calmodulin (CaM)-dependent kinase II (CaMKII) simultaneously interacts with multiple CaMKII-associated proteins (CaMKAPs) through its dodecameric structure. One study tested whether CaMKII undergoes LLPS with the carboxy-terminal tail of one of its associated CaMKAPs, the NMDA receptor (NMDAR) subunit GluN2B (GluN2Bc)[78]. The mixture of CaMKII, GluN2B and calcium-free CaM did not undergo LLPS (see the figure, part **a**); however, when calcium was added, the proteins formed condensates within 30 s. Once formed, the condensate persisted — even after calcium was chelated with use of EGTA — as long as ATP was present during the initial incubation to enable T286 autophosphorylation. However, whether CaMKII indeed undergoes LLPS at the synapse remains to be determined.

The carboxy-terminal tail of TARPγ2, also known as stargazin (STG), serves as a proxy for the AMPA receptor (AMPAR) complex. When this carboxy-terminal tail (STGc) and the scaffolding protein postsynaptic density protein 95 (PSD95) were added to the mixture[78], STGc, GluN2B and PSD95 underwent LLPS, but CaMKII remained in the diluted phase in the absence of calcium. When calcium was also added to the mixture, CaMKII accumulated in the condensate. Intriguingly, STGc and PSD95 segregated into another phase inside the CaMKII–GluN2B condensate (see the figure, part **b**).

Super-resolution microscopy has revealed that AMPARs and NMDARs exist in distinct nanodomains of the synapse[132,133]. Blockade of CaMKII–GluN2B disrupted LLPS and reduced segregation of AMPARs and NMDARs[78]. Accordingly, LLPS of CaMKII might mediate not only the accumulation of CaMKII beneath the synaptic contact but also the formation of glutamate receptor nanodomains at the surface.

The dodecameric structure of CaMKII, and its abundance, which exceeds that of GluN2B at the synapse, enables other proteins to join the condensate as follower proteins, thereby enriching them at the synapse as well. In this way, LLPS of CaMKII serves as a mechanism to translocate to defined nanodomains in the synapse not only CaMKII itself but also other proteins that bind CaMKII, such as densin 180 and α-actinin[186] (FIG. 4). Notably, in this context, the CaMKAP α-actinin has a central role in anchoring PSD95 and thereby AMPARs in spines[187] in a dynamic manner. Accordingly, NMDAR-mediated calcium-ion influx induces binding of the calcium–CaM complex to PSD95, displacing PSD95 from α-actinin[188]. This displacement causes PSD95 to diffuse out of spines[188]. Because PSD95 anchors AMPARs at postsynaptic sites, this loss of PSD95 leads to a loss of AMPARs from spines, as observed during homeostatic synaptic downscaling[188] and long-term depression[189]. That α-actinin also binds CaMKII might be important for the CaMKII-mediated phosphorylation of S73 of PSD95, which contributes to the displacement of PSD95 from spines upon prolonged Ca^{2+} influx[190].



Scale bar, 10 µm. DIC, differential interference contrast. Figure adapted from REF.[78], Springer Nature Limited.

Antiparallel coiled coil
A protein structure formed by two α-helices facing each other side by side in an antiparallel way; for example, by one helix oriented amino terminus to carboxy terminus and the adjacent helix oriented carboxy terminus to amino terminus.

Hebbian LTP
Long-term potentiation (LTP) that occurs when postsynaptic activity is paired with presynaptic activity.

Virus-like capsid
A protein structure that resembles the capsid structure that surrounds the viral genome.

binding, through LLPS or by signalling beyond the NMDAR complex.

When a CaMKII subunit is bound to the C-terminal tail of GluN2B, the binding protects CaMKII from reclosure, maintaining the constitutive activity CaMKII on other substrates[42]. Because this binding facilitates the CaMKII catalytic activation, rather than competing, the GluN2B–CaMKII interaction has been hypothesized to occur in a binding pocket different from the substrate-binding pocket[42,89]. This site has been named the 'T-site' (after the basal-state T286-docking site) as opposed to the substrate-binding site ('S-site')[42,89]. However, work showing the crystal structures of the CaMKII kinase domain bound to different effector proteins suggests that the core CaMKII-binding sequence of the GluN2B C-terminal tail binds to a rather extensive continuous region on CaMKII that includes both S-site and T-site residues simultaneously[46] (BOX 1). The same binding scheme is shared by other substrates and interacting proteins[46,76,77]. Thus, multiple CaMKAPs and substrates compete for the same binding sites. From the observed crystal structure[46], it is unlikely that the T-site and the S-site are completely distinct.

It is also not clear how GluN2B–CaMKII interaction facilitates catalytic activity of CaMKII, because the binding occupies the catalytic core. It is possible that CaMKII binding to GluN2B can facilitate the catalytic activity of the neighbouring subunit in the holoenzyme. The autoinhibitory regulatory region of CaMKII (residues 273–317) forms a helix, and the helices of two adjacent subunits can form an antiparallel coiled coil[22], which masks substrate-binding pockets. GluN2B binding to one of the substrate-binding pockets dissociates the coiled coil, possibly affecting the stability of the structure. This might impair the inhibitory activity of the other helix in the dimer *in trans*, and thus facilitate substrate binding. However, this model does not explain why GluN2B and TIAM1 can promote and maintain activity of CaMKII, whereas other substrates inhibit the kinase activity of CaMKII. In addition, electron microscopy has shown that most of the kinase domains are monomeric and less than 20% are dimeric[16]. Obviously, further refinements and validation are needed to address how CaMKII–GluN2B binding promotes CaMKII activity.

Proteasome. The proteasome is a large protein complex that mediates protein degradation. It accumulates in stimulated spines during LTP and is thought to remove regulatory proteins as well as structural proteins that would otherwise hamper the remodelling of spines, to enable spine growth[71]. The proteasome binds to CaMKII, and its activity-driven postsynaptic accumulation requires CaMKII binding to GluN2B[71,72]. One protein that is degraded by the GluN2B–CaMKII-anchored proteasome is ephexin 5, which is a RHOA GEF that counteracts spine formation[103]. Other potential targets of the GluN2B–CaMKII-anchored proteasome include ARC[104,105], the GTPase-activating proteins RAP1 and RAP2 and their upstream activator RAPGEF4 (also known as EPAC2)[106,107], the cyclin-dependent kinase CDK5 and the Polo-like kinase PLK2 (REF.[108]). These proteins typically limit or reduce postsynaptic strength or spine size.

Reciprocal kinase–effector complex with TIAM1. TIAM1, which promotes F-actin elongation by acting as a GEF and thereby an activator for the small G protein RAC, interacts with CaMKII in a manner similar to GluN2B during LTP[44]. It associates with CaMKII in a Ca^{2+}–CaM-dependent manner, and this binding locks CaMKII in an open, active state for an extended time even after calcium is removed, similarly to GluN2B. In this way, TIAM1 can activate CaMKII independently of T286 phosphorylation.

During binding, TIAM1 is phosphorylated at multiple sites by CaMKII. This phosphorylation activates the GEF activity of TIAM1 (REF.[109]). Thus, these two molecules potentially generate a reciprocally activating loop through binding as long as the complex persists. This mechanism, termed 'reciprocal activation within a kinase–effector complex', can convert a transient calcium signal into persistent activation of both CaMKII and the downstream molecule. A FRET-sensor study measuring the dynamics of the reciprocally activating kinase–effector complex[44] revealed that this complex can be maintained in spines for about 30 min after LTP induction, longer than the bulk CaMKII activation. Thus, reciprocal activation within a kinase–effector complex may be a general mechanism to maintain local CaMKII signalling for an extended period.

ARC. Strong activation of neurons induces transcription of the immediate-early gene *Arc*. ARC accumulates in non-stimulated spines, but is extruded from spines undergoing LTP, and the levels of ARC are inversely correlated with surface GluA1 expression[70]. The accumulation of ARC in non-stimulated spines requires ARC–CaMKIIβ interaction, which is diminished by increased Ca^{2+}–CaM levels as occurs in stimulated spines. Thus, the high calcium levels during LTP probably reduce CaMKIIβ–ARC interactions, reducing the localization of ARC in stimulated spines.

As ARC can promote the endocytosis of AMPARs[104,105], this so-called inverse tagging of non-stimulated spines with ARC may increase the contrast of synaptic weights between stimulated spines that are undergoing Hebbian LTP and the surrounding non-stimulated spines that experience a modest but general downscaling. Some work indicates that experience-dependent plasticity induces this inverse tagging mechanism to sharpen response tuning of cortical neurons[110]. Remarkably, ARC forms a virus-like capsid that is transmitted between cells[111,112]. Whether the inverse tagging requires assembly of a capsid structure and its transmission, and whether this transmission mediates downscaling of synapses that surround capsid-release sites as a potential mechanism of downscaling synapses formed by other, nearby neurons are important questions for future study.

Molecular mechanisms of signalling

Role of kinase activity. Early evidence implicating CaMKII in LTP was provided by postsynaptic expression or acute injection of a constitutively active mutant

form of CaMKIIα called 'tCaMKII', which contains the truncated amino acid sequence 1–290 and thus no regulatory region. Both approaches resulted in an increase in AMPAR-mediated excitatory postsynaptic potential (EPSC) amplitude and occlusion of LTP[113,114]. Although spine size correlates well with synaptic strength (reviewed in REF.[115]) and typically increases during LTP[48,62], overexpression of tCaMKII increased EPSC amplitude but not spine size[116]. Further in-depth analysis of the effects of expression of various CaMKII mutants on spine size versus EPSC amplitude revealed a remarkable bifurcation of these two parameters at the level of CaMKII (REF.[116]). Specifically, CaMKII binding to GluN2B can drive spine enlargement without requiring its catalytic activity, whereas an increase in postsynaptic AMPAR activity requires catalytically active CaMKII but potentially not its binding to GluN2B, as discussed below.

CaMKII mutants that can bind to GluN2B and form dodecamers invariantly increase spine size. Such mutants include the K42R–T286D double mutant, which combines a kinase-null mutation and an autophosphorylation-mimetic mutation, mimicking the active open conformation but without catalytic activity[116]. These findings suggest that CaMKII acts as a structural protein to augment spine size independently of its catalytic activity, whereas its kinase activity mediates T286 autophosphorylation, which should prolong binding to GluN2B in intact cells and thereby potentially spine enlargement. Whereas K42R–T286D double mutant and also T286D single mutant CaMKII increased spine size, they decreased EPSC amplitude[116]. By contrast, the T286D–T305A–T306A triple mutant increased both spine size and EPSC amplitude[116]. These results can be best explained by assuming that the T286D single mutant autophosphorylated itself on T305 or T306 even under basal, non-stimulated conditions[117], which blocked Ca^{2+}–CaM binding and thereby full activation of CaMKII (REFS.[50,55,118]). The T286D–T305A–T306A combination of mutations prevented autophosphorylation of T305 and T306 such that the kinase could be fully activated by Ca^{2+}–CaM, resulting in increased EPSC amplitude. Thus, to increase EPSC amplitude, CaMKII has to be able to be fully activated through Ca^{2+}–CaM binding to the autoinhibitory domain (or through the removal or absence of the autoinhibitory domain, as in tCaMKII).

Nonetheless, an increase in EPSC amplitude was observed with T286D when it was expressed in the presence of the NMDAR antagonist APV[116]. Under these conditions, calcium influx through the NMDAR should be blocked, and so Ca^{2+}–CaM would be expected not to be available to bind to the T305–T306 region to fully activate CaMKII. This consideration raises the question of why phosphorylation at T305 and T306, as presumably occurs in the T286D single mutant, prevents an increase in EPSC amplitude at all. A potential answer is that other sources of calcium could drive CaMKII activation under basal, non-stimulated conditions to augment synaptic strength upon expression of the T286D-mutant CaMKII, without triggering phosphorylation of T305 or T306. For example, LTP induced by a 5-Hz tetanus that is 3 min long with simultaneous activation of the β_2-adrenergic receptor depended on calcium influx through the $Ca_V1.2$ channels only and not at all through NMDARs[119]. However, this LTP still also required CaMKII binding to GluN2B (H. Qian and J.W.H., unpublished results). Thus, blocking calcium influx through NMDARs seems to have prevented phosphorylation at T305 and T306 of the T286D-mutant CaMKII, enabling activation of this mutant in a manner that drives an increase in EPSC amplitude. Perhaps under basal neuronal-activity conditions, NMDAR-mediated calcium influx is more effective than $Ca_V1.2$-mediated influx in stimulating T305/T306 phosphorylation and thus preventing an increase in AMPAR activity. However, $Ca_V1.2$-mediated influx may be sufficient to enable temporary activation of CaMKII to drive the increase in EPSC amplitude seen when T286D-mutant CaMKII is overexpressed with APV present.

Single-cell, CRISPR-mediated knockout of the gene encoding CaMKIIα (which is three times more abundant than CaMKIIβ in the rat forebrain[120]), either alone or in combination with knockout of the gene encoding CaMKIIβ (double knockout) in the hippocampal CA1 region, reduces the AMPAR-mediated EPSCs by about 50% and NMDAR-mediated EPSCs by about 30%[43]. These results imply that, under basal conditions (that is, with no specific treatment of the rodents or slices besides routine handling), development of full synaptic strength depends on CaMKII.

Furthermore, LTP is abolished in the CaMKIIα-knockout and double-knockout neurons[43]. Knockout of only CaMKIIβ also impairs LTP but not the basal transmission of AMPAR-mediated EPSCs[43]. In double-knockout neurons, restoration of expression of CaMKIIα fully rescued AMPAR and NMDAR EPSCs and LTP, whereas restoration of expression of CaMKIIβ rescued only NMDAR-mediated EPSCs. Accordingly, CaMKIIα is important for both basal postsynaptic AMPAR activity and LTP, whereas CaMKIIβ is dispensable for basal AMPAR activity but is important for NMDAR function and LTP. The specific roles of T286 phosphorylation, the kinase activity of CaMKII and the interaction of CaMKII with GluN2B and other substrates were tested by overexpression of various mutant forms of CaMKIIα (including the phospho-dead T286A and the kinase-dead K42R) on the double-knockout background. This approach revealed that both T286 phosphorylation and kinase activity of CaMKIIα are necessary for rescuing AMPAR current and LTP, but not for rescuing NMDAR current. These results[43] imply that a structural role of CaMKII is sufficient to support the functional availability of NMDARs at postsynaptic sites, whereas catalytic activity is required to augment AMPAR current.

This requirement for catalytic activity to increase AMPAR-mediated currents could reflect the need to phosphorylate other substrates, such as transmembrane AMPAR regulatory proteins (TARPs; for example, TARPγ8 (REFS.[88,121])) or RHO GTPase protein-activating GEFs (such as kalirin 7 and TIAM1) that augment F-actin formation and thereby spine growth to provide the structure for an increase in postsynaptic AMPAR content (see later for discussion of RHO regulation)[44,122–124]. Alternatively, CaMKII might need to maintain its own phosphorylation of T286 for continued interactions with other postsynaptic proteins, especially

Myristoylated
Bearing a lipid moiety known as a myristoyl side chain.

GluN2B. Mutations at the extended substrate-binding pocket (F98K and I205K; BOX 1) that prevent CaMKII binding to GluN2B and other CaMKAPs also impair LTP and basal AMPAR current[43]. Thus, overall, T286 phosphorylation, kinase activity and substrate or GluN2B binding are all required for the maintenance of basal AMPAR activity and LTP.

Why does single-cell knockout[43] but not system-wide knockout of CaMKIIα[18] decrease AMPAR activity? Also, single-point mutations that disable autophosphorylation (T286A in CaMKIIα)[125] or kinase activity (K42R) do not change basal AMPAR-mediated synaptic function[126] in respective knock-in mice, whereas NMDAR-mediated transmission can be altered in these animals[127]. Furthermore, double knockout of CaMKIIα and CAMKIIβ in adult mice (8–10 weeks old) using a Cre-dependent conditional knockout did not change basal transmission, but completely abolished LTP[128]. Perhaps CaMKII facilitates synaptic strengthening through trans-synaptic mechanisms. Synapses in a knockout neuron could be at a collective disadvantage and 'lose out' to other synapses formed nearby that might become stronger because resources of the presynaptic input neurons might be allocated to the synapses that they form with surrounding neurons. Consistently, presynaptic and postsynaptic parameters of synaptic strength are strongly correlated (reviewed in REF.[115]), and overexpression of CaMKIIβ increases presynaptic glutamate release probability, whereas its knockdown prevents homeostatic synaptic upscaling induced by chronic inhibition of transmission[129].

Pharmacological reversal of LTP and AMPAR basal transmission. That basal AMPAR activity and LTP are both affected by impaired CaMKII binding to GluN2B supports the idea that, in vivo, LTP mechanisms maintain synaptic strength. This notion is supported by effects of acute application of membrane-permeant, CaMKII-inhibiting peptides, such as tatCN21, which is derived from the CaMKII-inhibitory protein CaMKIIN1[130]. Application of 5 μM tatCN21 blocked CaMKII and induction of LTP, but this dose of tatCN21 did not reverse LTP when applied after LTP had already been induced[25]. By contrast, application of 20 μM tatCN21 displaced CaMKII from GluN2B, reversed LTP and reduced basal synaptic transmission[40], suggesting that CaMKII–GluN2B binding increases postsynaptic AMPAR activity.

myr-CN27, which expands the CaMKII-binding sequence of tatCN21 by six residues, and myr-AIP2, which is derived from the autoinhibitory domain of CaMKII (REFS.[130,131]), are two new peptides that share the same binding site on CaMKII with tatCN21 but that are myristoylated. The application of 1 μM myr-CN27 and myr-AIP2 reduced basal transmission and reversed LTP[41]. The simplest explanation for the different effects of 5 μM tatCN21 versus 1 μM myr-CN27 and myr-AIP2 is that myr-CN27 and myr-AIP2 also displace CaMKII from GluN2B with higher potency than does tatCN21. Extensive controls for myr-CN21 and myr-AIP2 ruled out off-target presynaptic effects that have been reported for tatCN21 (REF.[41]). Prolonged, but not brief, activation of the CaMKII inhibitor paAIP2 (10–50 μM estimated intracellular concentration[37]) also reduced LTP maintenance and basal transmission[41], further suggesting that CaMKII–CaMKAP interactions are crucial for LTP maintenance and basal transmission[37].

Nanodomain signalling around the GluN2B–CaMKII complex and synaptic plasticity. Imaging studies suggest that CaMKII activation and accumulation are restricted to those spines that experience potentiation[34,93]. Furthermore, CaMKII is necessary for standard LTP in CA1, and LTP is impaired when CaMKII binding to GluN2B is abrogated[88]. Thus, activity-dependent CaMKII binding to GluN2B seems to be important for the synapse specificity of LTP, a prerequisite for the role of LTP in information storage, by recruiting CaMKII to those synapses that undergo LTP.

How can GluN2B-associated CaMKII augment the functional availability of AMPARs at postsynaptic sites when localized at NMDAR nanodomains, which only partially overlap with AMPAR nanodomains?[132,133] NMDAR-anchored CaMKII might phosphorylate nearby AMPARs that then become trapped when diffusing through AMPAR nanodomains. Consistent with this, activity-induced phosphorylation of GluA1 on S831 and postsynaptic accumulation of auxiliary AMPAR TARP subunits was impaired in knock-in mice that express GluN2B$^{L1298A–R1300Q}$ and showed reduced CaMKII–GluN2B binding[88]. S831 phosphorylation augments single-channel conductance of AMPARs[134,135], which might contribute to an increase in postsynaptic response during LTP induction[136]. Alternatively, this increase in postsynaptic response could be mediated by recruitment of GluA1 homomeric AMPARs[137] (see also REF.[138]), which have a higher conductance than do GluA2-containing AMPARs[139]. However, LTP is normal in knock-in mice expressing GluA1^{S831A}, suggesting that S831 phosphorylation is not strictly necessary[140]. Also, S831 phosphorylation is not necessary for tCaMKII-induced translocation of GluA1 to the synapse[141,142]. Indeed, less than 1% of GluA1 is phosphorylated, even after chemical LTP induction[143] (but see REF.[142]). Therefore, the relevance of S831 phosphorylation is not fully clear.

A lasting increase in postsynaptic AMPAR function could be through phosphorylation of the auxiliary AMPAR subunit TARPγ2 (REFS.[144,145]) or TARPγ8 (REF.[121]) (FIG. 5). TARPs mediate postsynaptic localization of AMPARs by binding to PSD95 and its homologues PSD93 and synapse-associated protein 102 (SAP102; also known as DLG3)[145–148]. TARP phosphorylation by CaMKII augments TARP binding to PSD95 (REFS.[144,145]), trapping AMPARs at postsynaptic sites[121,149] (but see REF.[150]). However, further analysis is required to address disparate findings that implicate CaMKII-mediated phosphorylation of TARPγ2 (REF.[151]) versus TARPγ8 (REF.[121]) in the hippocampus. The C-terminal intracellular domains of TARPs contain clusters of positively charged arginine residues (R225 to R250 in mouse TARPγ2, and R261 to R288 in TARPγ8), and multiple phosphorylated serine residues have been identified in and around this region[151]. Two of these residues in TARPγ8 (S277 and S281) seem to be crucial CaMKII targets; although mice

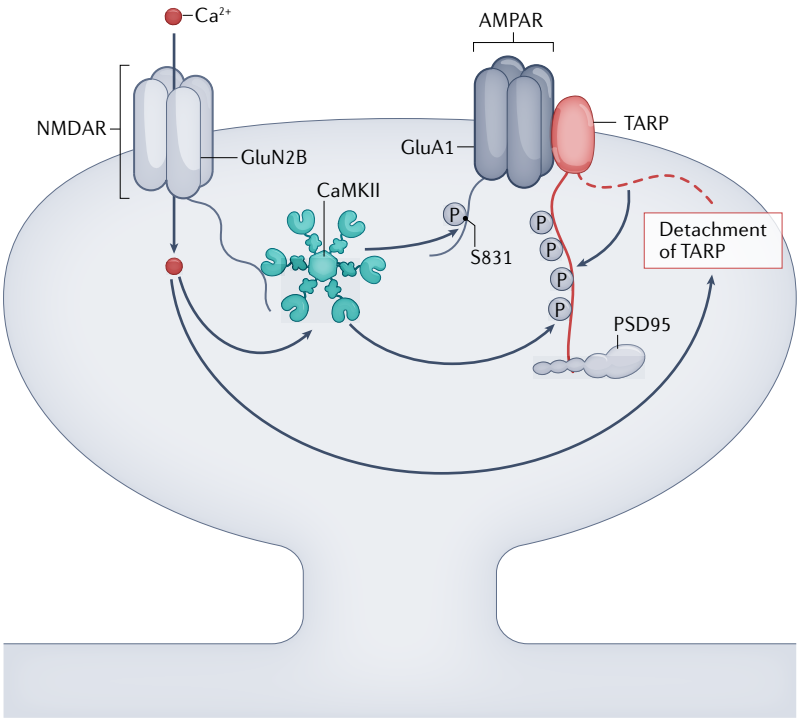


Fig. 5 | **Role of CaMKII binding to NMDA receptor subunit 2B in regulating AMPA receptors.** Calcium influx through NMDA receptors (NMDARs), as occurs during long-term potentiation, triggers binding of calcium–calmodulin (CaM)-dependent kinase II (CaMKII) to NMDAR subunit 2B (GluN2B). At this location, CaMKII can effectively phosphorylate nearby AMPA receptors (AMPARs) — at S831 in the GluA1 subunit of the receptor — and their auxiliary subunits known as transmembrane AMPAR regulatory proteins (TARPs). Phosphorylation of S831 of GluA1 increases AMPAR conductance. Calcium also promotes detachment of the cytosolic carboxy termini (C termini) of TARPs, which have multiple positively charged arginine and lysine residues, from the plasma membrane. The detachment enables CaMKII to phosphorylate multiple residues in the TARP C termini; phosphorylation of S277 and S281 in TARPγ8 has been specifically implicated as important for long-term potentiation[121]. These phosphorylations reduce the net positive charge of TARPγ8 and thereby reduce the reassociation of the TARP C termini with the plasma membrane. In this way, the number of TARP C termini that are available for binding to PDZ domains of postsynaptic density protein 95 (PSD95) is increased, enabling more trapping of AMPAR–TARP complexes at postsynaptic locations[149].

expressing TARPγ8 with these residues replaced with alanine show normal basal synaptic transmission, they show impaired LTP, learning and memory[121]. Therefore, CaMKII may mediate LTP partly by phosphorylating TARPs (FIG. 5).

Under basal conditions, TARP C termini are associated with the plasma membrane, owing to electrostatic interactions between their multiple positively charged arginine residues (which are part of the CaMKII consensus phosphorylation sites) and negatively charged phospholipids[144,145,152]. Because calcium ions disrupt such electrostatic interactions, NMDAR-mediated calcium influx may make TARPs more accessible for CaMKII, by decreasing association of their C termini with the plasma membrane[144,145,152]. TARP phosphorylation by CaMKII prevents reassociation of TARP C termini with the plasma membrane and thus augments increases in TARP–PSD95 binding[144,145] and postsynaptic AMPAR anchoring. Consistent with this notion, chemically induced LTP increases the content of TARPγ8 and other TARPs in PSD preparations[88].

LTP is impaired in mice expressing S277A and S281A double-mutant TARPγ8, but not when all endogenous AMPARs are replaced with a GluA1–TARPγ8 fusion protein with these S277A/S281A mutations[150]. Conversely, postsynaptic AMPAR localization is disrupted when the four C-terminal residues of TARP-γ8, TTPL, which mediate PDZ binding, are removed from the GluA1–TARPγ8 fusion protein[150] but not when they are removed from endogenous TARPγ8 (REF.[153]). Perhaps the C terminus of GluA1-fused TARPγ8 has a higher propensity than the free TARPγ8 to detach from the plasma membrane and thus to bind to PSD95, independent of its CaMKII-mediated phosphorylation (FIG. 5). That TTPL is not required for (non-fusion) TARPγ8 to localize AMPARs[153] could be explained by a second, electrostatic interaction site between TARPs and the first PDZ of PSD95 (REF.[154]).

CaMKII also mediates LTD. CaMKII decreases postsynaptic AMPAR content by phosphorylating GluA1 on S567 (REFS.[10,155]). LTD does not depend on CaMKII–GluN2B binding[88], which is prevented during LTD by another Ca^{2+}–CaM-dependent kinase, death-associated protein kinase 1 (DAPK1), whose binding site on GluN2B overlaps with that of CaMKII (REF.[156]). DAPK1 blocks CaMKII binding during modest, LTD-driving but not large, LTP-driving calcium influx[156]. In vitro, increasing Ca^{2+}–CaM concentrations cause a shift from GluN2B–DAPK1 binding, which is impaired by Ca^{2+}–CaM, to GluN2B–CaMKII binding, which is induced by Ca^{2+}–CaM[42,157] (at a half-maximal effective concentration of about 300 nM (REF.[156])). In addition, during LTD, the phosphatase calcineurin, which is activated at lower Ca^{2+}–CaM levels than CaMKII is, dephosphorylates DAPK1 on S308, which promotes DAPK1 binding to GluN2B[156]. However, mice that express a mutant version of GluN2B that is deficient in CaMKII binding show LTD that is insensitive to DAPK1 inhibition[156], implying that other CaMKII-mediated mechanisms in addition to DAPK1–CaMKII competition are at work during LTD, at least in these mice.

The modest but prolonged increase in Ca^{2+}–CaM levels during LTD induction also promotes CaMKII autophosphorylation of T305 and T306, which is required for LTD but not LTP[51]. This phosphorylation is slow in vitro and more prominent during LTD-inducing prolonged but low-frequency tetani than during LTP-inducing short-duration but high-frequency tetani (100 Hz for 1 s), as it requires the slow unbinding of Ca^{2+}–CaM from CaMKII. The phosphorylation of T305 and T306 reduces CaMKII–NMDAR binding[157] and increases autonomous CaMKII activity[51], which could especially apply to phosphorylation sites in CaMKII target proteins that are important for LTD. LTD-inducing but not LTP-inducing stimuli also increase the phosphorylation of GluN2B on S1303 (in the GluN2B–CaMKII-binding site) by DAPK1 (REF.[158]), decreasing CaMKII–GluN2B binding[159,160]. Accordingly, the modest but long-lasting increase in Ca^{2+}–CaM levels during LTD induction can distinguish LTD from LTP by impairing CaMKII binding to GluN2B through supporting the autophosphorylation of CaMKII on T305 and T306 and through phosphorylation of GluN2B on S1303 by DAPK1.

Consensus phosphorylation sites
Amino acid sequences that are recognized by specific protein kinases as substrate sites.

Half-maximal effective concentration
The concentration of a molecule at which it exhibits half-maximal efficacy with respect to, for example, enzymatic activity or binding interactions.

Retromer complex
The complex formed by the vacuole protein sorting gene products VPS26, VPS29 and VPS35.

Spine pruning
Loss of spines or reduction in spine number over time in the brain, especially during development but also later in life.

Role of CaMKII in NMDAR-dependent metaplasticity. LTP can induce a rapid switch (within 5 min) from mostly GluN2B-containing NMDARs to those containing GluN2A or GluN2A plus GluN2B at potentiated postsynaptic sites[161]. CaMKII phosphorylates GluN2A on S1459, five residues upstream of its C terminus, which constitutes a PDZ domain binding motif[162,163]. S1459 phosphorylation of GluN2A augments the binding of sorting nexin 27 (SNX27) to the PDZ domain, and SNX27 in conjunction with the retromer complex enhances surface expression of GluN2A during chemically induced LTP[162,163]. This phosphorylation also increases calcium influx through NMDARs[163], potentially creating a positive feedback loop to keep CaMKII bound to GluN2B (as part of heterotrimeric GluN1–GluN2A–GluN2B NMDARs[139]). In addition, CaMKII recruits the casein kinase CK2 to GluN2B, which phosphorylates on S1480 to impair PSD95 binding and surface expression of GluN2B[164], potentially leading to a selective loss of GluN1–GluN2B heterodimeric NMDARs. Also, the switch from GluN2B to GluN2A is reduced in CaMKIIα T286A knock-in mice, implicating prolonged CaMKII activity or CaMKII–GluN2B binding in this process[127].

Regulation of small GTPases and actin cytoskeleton. F-actin is the main cytoskeletal component in spines and governs spine size and LTP[61,92,124,165]. Formation of F-actin is controlled by the small GTPases CDC42, RHOA and RAC1, which are regulated by CaMKII (REFS.[44,124,165,166]). Postnatal deletion of *Cdc42* or *Rac1* from excitatory neurons impairs LTP, sLTP and learning, implicating the regulation of actin by these GTPases in synaptic plasticity[124,167,168]. CaMKII supports the release of brain-derived neurotrophic factor (BDNF) from stimulated spines, and autocrine activation of the BDNF receptor TRKB on the same spine stimulates CDC42 and RAC1 (REFS.[124,166]). These RHO-family GTPases are activated by GEFs and inactivated by GTPase-activating proteins. The RAC-specific GEFs kalirin 7, TRIO and TIAM1 are important for sLTP and LTP, linking CaMKII signalling and RAC activation[44,123]. Activated RAC stimulates the serine–threonine kinase PAK, which phosphorylates and activates LIM domain kinase 1 (LIMK1). LIMK1, in turn, inactivates the actin-depolymerizing protein cofilin by phosphorylating it at S3, leading to the stabilization of F-actin in the spine head[169]. RAC also couples to WAVE, which activates the actin-related protein 2 (ARP2)–ARP3 complex, which nucleates and branches F-actin filaments[169].

CaMKII also regulates the small GTPase RAS during LTP[170,171] by phosphorylating SYNGAP, a RAS GTPase-activating protein concentrated in the PSD[172,173]. SYNGAP phosphorylation triggers its dispersal from PSDs and spines, disinhibiting RAS[174], which in turn activates extracellular signal-regulated kinases (ERKs)[170,175] and supports sLTP[174]. Notably, close relatives of RAS — RAP1 and RAP2 — have roles in LTD and reversal of LTP (depotentiation)[106,170,176]. The signalling seems to occur in specific nanodomains, as determined by dominant-negative mutants targeted to different subcellular domains[106].

Whereas CDC42 shows spine-specific activation, RAC1, RHOA and RAS signals spread out of the stimulated spine and into surrounding spines 5–15 µm away (REFS.[124,165,171,177]). This spread does not cause LTP or enlargement in surrounding spines by itself, but instead can facilitate LTP when combined with the stimulation of these spines[124,165,171]. Thus, LTP probably depends on the concerted regulation of these GTPases, and activation of a single one is insufficient[124].

Photomanipulation of CaMKII signalling

Electrical or chemical stimulation activates many kinases in addition to CaMKII, complicating interpretation of the actions of CaMKII in studies using such manipulations. To resolve this problem, a photoactivatable CaMKII has been developed[178]. Blue light-activated light–oxygen–voltage-sensing domain 2 was fused to the hinge region of CaMKII, inducing a catalytically active open conformation of CaMKII, which triggers spine enlargement and LTP. Unlike for glutamate uncaging-induced sLTP, this spine enlargement is protein synthesis dependent. Apparently, the stimulation of glutamate receptors renders LTP protein synthesis independent through unknown mechanisms that are not engaged by CaMKII activation alone.

Interestingly, photoactivatable CaMKII activates spine CDC42, but not RHOA, although glutamate uncaging leads to activation of RHOA via CaMKII. Thus, RHOA activation in response to glutamate uncaging may require other calcium-dependent processes in addition to CaMKII, which may indirectly regulate RHOA. RHOA signalling is involved in spine pruning and shrinkage, whereas RAC1 and CDC42 promote spine maturation[169]. Given that spine structure under basal conditions is stable over days[179], RHOA may destabilize spines so that they can undergo structural changes during synaptic plasticity[180].

To link the timescales of CaMKII action in synapses with the timescale of behaviour, it is necessary to manipulate CaMKII in a temporally controlled manner. Traditionally, this has been done using pharmacological tools. For example, in the contextual fear-conditioning paradigm, when tatCN21, which crosses the blood–brain barrier, is injected before conditioning, the memory tested 24 h later is impaired[25]. However, there is no effect on fear memory when tatCN21 is injected immediately before the memory test. This suggests that CaMKII activity is required for formation of this memory, but not for its maintenance or recall[25]. By contrast, viral transduction of dominant-negative CaMKII after establishment of conditioned place avoidance memory extinguished this memory, suggesting a role of CaMKII in the maintenance of this memory[181]. However, one must bear in mind that such manipulations in vivo have limited temporal resolution (hours for pharmacological compounds, and days for viruses), making it difficult to distinguish between loss of synaptic plasticity and any adaptations caused by the manipulation. Furthermore, pharmacological tools are not cell type specific. Also, it is unclear whether the absence of an effect on fear-memory recall by tatCN21 injection in vivo is perhaps because the concentration of this peptide in the relevant neurons is

Inhibitory avoidance task
Behavioural task in which an animal is trained to avoid a situation or localization.

sufficient to inhibit CaMKII activity but not high enough to displace CaMKII from GluN2B, with the latter action but not former action being expected to reverse LTP[40] and thereby impair memory.

By contrast, the photoinducible CaMKII inhibitor paAIP2 enables determination of the timescale, cell types and brain region of global CaMKII action for specific behavioural adaptations, including learning and memory, with much higher temporal resolution[37,182,183]. The temporal window of CaMKII action required in excitatory neurons of the amygdala for the inhibitory avoidance task was examined with use of paAIP2. Memory formation in the task was impaired when CaMKII was inhibited by illumination of paAIP2-expressing neurons during, but not 3–5 min after, training[37]. Accordingly, CaMKII activation during, but not after, training is necessary to form avoidance memory.

Synaptic plasticity in the motor cortex seems to be crucial for motor skill learning. However, it has been difficult to assess which neurons undergo the synaptic plasticity that is relevant to motor skill learning. In one study, mice were trained to walk on a moving treadmill using only their front limbs, while their rear limbs were fixed[182]. This behavioural plasticity required disinhibition of the activity of pyramidal neurons in the motor cortex by somatostatin-positive (SST^+) interneurons during learning. When CaMKII activity was inhibited (with use of paAIP2) in the pyramidal neurons during training, both circuit plasticity and learning were inhibited. These results suggest that CaMKII-dependent synaptic plasticity in pyramidal neurons is required for this form of motor skill learning, and that SST^+ interneurons regulate the synaptic activity and, thereby, the synaptic plasticity of those pyramidal neurons.

In *Drosophila*, paAIP2 was used to measure the role of CaMKII in the timing of behavioural motivation[183]. Accordingly, CaMKII activity in four male-specific neurons (Crz^+ neurons) determines the duration of copulation. Early in mating, the male persists through even life-threatening stimuli, but his persistence declines over the course of 15–20 min, until even mild challenges cause him to flee. Using heat as a life-threatening stimulus together with paAIP2, the authors measured the temporal window of CaMKII activation during this motivational shift. This approach revealed that CaMKII is activated at the onset of mating, presumably by an increase in intracellular calcium concentration. Also, the Camui FRET sensor revealed that CaMKII activity lasts for about 6 min, much longer than the elevation in intracellular calcium concentration. Active CaMKII prevents the motivational shift by life-threatening stimuli (such as heat) during this time window. How CaMKII physiologically affects Crz^+ neurons is to be determined, but these experiments suggest that calcium signalling may be inhibited by CaMKII activation[183]. Overall, this work suggests that CaMKII in Crz^+ neurons acts as a timer of motivational shift.

Conclusions and perspective

CaMKII received early attention in postsynaptic signalling because of its abundance, unusual structure and presumed self-perpetuating autoregulation. Recent work has defined potential key postsynaptic CaMKII substrates, including TARPs and RAC and RHO GEFs, and has highlighted the importance of the role of CaMKII as a structural protein that tunes itself through autophosphorylation towards binding various CaMKAPs. However, much work lies ahead of us with respect to defining the molecular mechanisms of synaptic plasticity and learning. Central to such future work will be the development of new tools, especially to define how localizing CaMKII to nanodomains supports synaptic plasticity. We anticipate the creation of tools that specifically allow targeting of CaMKII and reporters of its activity to NMDARs and other postsynaptic anchoring proteins for CaMKII to elucidate details of nanospace signalling. Such new technologies will allow us to further define the numerous and diverse functions of CaMKII in health and disease.

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