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Mechanisms of CaMKII action in long-term potentiation

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Abstract

Long-term potentiation (LTP) of synaptic strength occurs during learning and can last for long periods, making it a probable mechanism for memory storage. LTP induction results in calcium entry, which activates calcium–calmodulin-dependent protein kinase II (CaMKII). CaMKII subsequently translocates to the synapse, where it binds to the NMDA-type glutamate receptors and produces potentiation by phosphorylating principal and auxiliary subunits of AMPA-type glutamate receptors. These processes all are localized to stimulated spines and account for the synapse specificity of LTP. In the later stages of LTP, CaMKII has a structural role in enlarging and strengthening the synapse.

Introduction

Long-term potentiation (LTP) is a process whereby brief periods of synaptic activity can produce a long-lasting increase in the strength of a synapse, as shown by an increase in size of the excitatory postsynaptic current (EPSC). LTP, *in vivo*, can last for at least a month[1], and similar changes in synaptic strength occur during learning[2, 3]. When LTP is prevented by various mutations, memory is impaired[4]. Taken together, these findings indicate that LTP has an important role in learning and memory, and a major effort has been therefore made to understand the mechanisms underlying this process.

The CA1 region of the hippocampus, a brain region that is vital for long-term memory formation, has been used as the primary model system for understanding LTP[5]. The process of LTP at hippocampal CA1 synapses is initiated by brief periods of high-frequency presynaptic stimulation. Glutamate, the neurotransmitter that is released at these synapses, activates postsynaptic AMPA- and NMDA-type glutamate receptors (AMPARs and NMDARs). The opening of NMDARs during the induction of LTP leads to calcium entry that triggers a biochemical cascade, the end product of which is the long-lasting potentiation of the AMPAR-mediated EPSC.

Strong evidence now exists that calcium elevation initiates this biochemical cascade through activation of calcium–calmodulin-dependent protein kinase II (CaMKII), a major synaptic protein[6]. This kinase is a large holoenzyme consisting of 12 subunits. There are two types of

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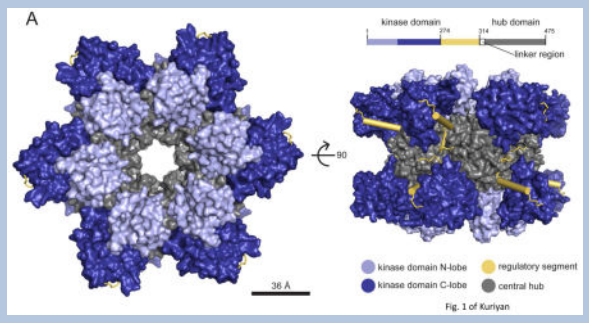
subunits (alpha and beta) both of which are capable of catalysis. Recent work has led to crystallization of the holoenzyme and the elucidation of how the enzyme's structure relates to its function (BOX 1)[7]. The kinase can act as a protein switch[8]; once activated by calcium–calmodulin, the enzyme can be autophosphorylated at T286 (on the alpha subunits; 287 on the beta subunits), an event that makes CaMKII activity persist even after the calcium concentration falls to baseline levels[9] (the T286 autophosphorylated state of CaMKII is termed the 'autonomous' state). If active CaMKII subunits or holoenzymes are introduced into CA1 neurons, the EPSC becomes large and LTP can no longer be induced by synaptic stimulation[10, 11]. If persistent activation of CaMKII is prevented by a knock-in mutation encoding T286A in CaMKII, LTP induction is prevented and mice show profound memory impairments, notably after one-trial learning [12, 13]. In many mutants with enhanced learning, CaMKII activation is enhanced[14]. Taken together, these results indicate that activated CaMKII is sufficient to trigger LTP and that CaMKII activation is necessary for LTP induction and certain forms of learning.

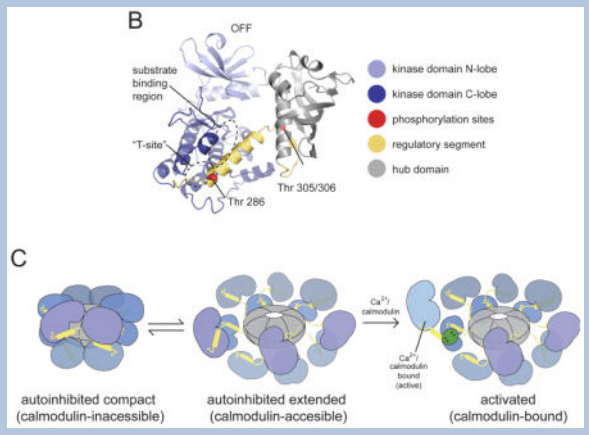
Box 1

Structural and functional properties of CaMKII

The architecture of the CaMKII holoenzyme has been determined on the basis of its crystal structure (see the figure, part **a**) [7]. The holoenzyme has 12 subunits, each with a carboxy-terminal association domain that forms a central organizing hub (grey) on to which the catalytic kinase domains (blue) fold into two rings of 6. These domains have a bi-lobed structure with the ATP binding site located between the two lobes. A regulatory segment (yellow) regulates the activity of the kinase domain (the S-site). A linker region connects the regulatory domain to the hub, which has a central pore of unknown function [146]. In the autoinhibited state, the regulatory segment forms an α-helix that blocks the catalytic S-site in a pseudosubstrate manner (see the figure, part **b**). This inactive state is further stabilized by sequestering the T286 phosphorylation site in a hydrophobic groove (the T-site) [147]. The crystal structure reveals a 'compact' state in which the portion of the regulatory domain that is recognized by calmodulin is sequestered into the association domain, making it impossible for calmodulin to bind. Activation is only possible because the compact state is in equilibrium with an extended state to which calmodulin can bind (see the figure, part **c**) [7, 148, 149]. The linker region varies in different CaMKII isoforms and determines the set-point of this equilibrium and, secondarily, the calcium level required for activation. Upon calmodulin binding, the regulatory segment is stripped from the S-site [7, 150], allowing substrate phosphorylation and starting the slower process of T286 intersubunit autophosphorylation. If T286 phosphorylation has not occurred, lowering calcium rapidly turns off activity; if T286 phosphorylation has occurred, activity remains (this is termed the 'autonomous' state) and the T-site is open for binding (for example, to the NR2B subunit of NMDA-type glutamate receptors). T286 phosphorylation dramatically strengthens the binding of calmodulin, a process called 'trapping'. The autonomous activity of CaMKII for phosphorylating various substrates is up to five times higher with calmodulin bound than it is when calmodulin is not bound[126]. Formation of autonomous CaMKII has a Hill coefficient of approximately eight with respect to calcium, indicating a virtual

threshold [151]. Various cooperative reactions underlie this ultrasensitivity. Four calcium binding events are required to fully activate calmodulin, which displays cooperativity when binding to the holoenzyme [152]. Calmodulin can activate a CaMKII subunit, but the subunit can only phosphorylate a neighbour if that subunit also has calmodulin molecule bound (as required to expose T286).





In this Review, we will describe recent progress in understanding the role of CaMKII in LTP. It has been shown that LTP occurs specifically at synapses that are stimulated (inactive synapses that are only microns away from a stimulated synapse remain unpotentiated). We will review recent data showing that activation of CaMKII following synaptic stimulation is synapse-specific and elucidating how this specificity is achieved. We will also describe experiments that have identified the downstream process by which activated CaMKII causes EPSC enhancement during the early stages of LTP. Finally, we will discuss experiments suggesting that CaMKII can contribute to the later stages of LTP, when structural changes in the synapse become important for long-term memory. As a result of space limitations, we will not discuss the presynaptic functions of CaMKII in LTP[15], the role of other kinases and second messengers in LTP[16], or the phosphatase-dependent processes that limit or reverse CaMKII-dependent activation[17].

Synapse-specific activation

Early experimental data suggested that LTP is a synapse-specific event[18, 19], and optical methods have now made it possible to demonstrate directly that this is correct. An identified spine can be stimulated using two-photon glutamate uncaging. Such uncaging gives rise to a

glutamate pulse with submicron dimensions that directly targets the identified spine and induces LTP at the synapse on that spine. Nearby synapses that are only microns away are not potentiated (FIG. 1)[20]. Given the complex biochemical cascade that is involved in LTP and the small distances that separate different synapses, it is remarkable that synapse-specific LTP can be achieved.

High-resolution imaging of CaMKII activation during LTP induction has been made possible by the development of the Camui activity sensor[21, 22]. This sensor comprises two different fluorophores — monomeric enhanced green fluorescent protein (mEGFP) and resonance energy-accepting chromoprotein (REAch) — that are attached to different ends of CaMKII subunits. The level of fluorescence resonance energy transfer (FRET) that occurs between the two fluorophores is an indicator of the distance between them: when the enzyme is activated, conformational changes increase the distance between the fluorophores and thus decrease the amount of FRET. Through use of this sensor and two-photon fluorescence lifetime imaging (2pFLIM), which allows quantification of FRET, the conformational change in CaMKII that is associated with activation can be monitored at the level of a single spine in real time. Moreover, such monitoring can be conducted while LTP is induced at the same spine using a second two-photon laser to uncage glutamate, which activates NMDARs on that spine. Using this approach, it can be seen that CaMKII is activated within seconds after the start of synaptic stimulation and that the activation is restricted to the stimulated spine (FIG. 1)[22]. At the end of stimulation, CaMKII activity decays to baseline levels within ~1 min. By contrast, when using a mutant variant of CaMKII (T286A) that cannot be autophosphorylated, the activity of the enzyme decays within seconds. Taken together, these results show that CaMKII activation has the localization that is required to account the for synapse specificity of LTP. The results also show autophosphorylation makes CaMKII activity persist for much longer than the duration of calcium elevation. The slow rate at which active CaMKII turns off (~1 min) means that during this period, the enzyme acts as a biochemical integrator of the multiple calcium pulses that generally occur during LTP induction. This integration is important for the full activation of CaMKII that is required to produce LTP.

Investigations have examined the mechanisms of CaMKII activation, and the results of these studies provide a framework for understanding why activation of the kinase is localized to the stimulated spine. The primary source of calcium entry into spines is through NMDARs. The opening of these channels requires both the presynaptic release of glutamate and strong postsynaptic depolarization, which relieves the magnesium blockade of these receptors[23]. These channel properties allow NMDARs to compute the Hebbian condition that is crucial for associative memory storage. Calcium indicators have been used to study calcium entry through NMDARs into spines, and such studies have confirmed that calcium elevation is localized to stimulated spines (FIG. 1)[22, 24–27].

Spines largely contain two types of NMDARs: those containing the NMDAR subtype 2A (NR2A) subunit and those containing the NR2B subunit[28]. NR2A-containing NMDARs open briefly but reliably following glutamate release, whereas NR2B-containing receptors have a lower probability of opening but continue to have many brief openings for several hundred milliseconds after glutamate release[29, 30]. When calcium enters through a NMDAR,

the concentration of calcium becomes high near the inner mouth of the channel, a region called a calcium nanodomain[31]. Calcium then diffuses into the bulk of the spine head within microseconds.

Experiments have been conducted to determine whether CaMKII activation depends on events that occur in the nanodomain or in the bulk of the spine head; these were based on the calcium-buffering capabilities of BAPTA, a fast calcium buffer, and EGTA, a slow calcium buffer (the steady-state dissociation constant is similar for both of these buffers). As calcium traverses the nanodomain quickly, only a fast buffer can block calcium elevation in the nanodomain, whereas either a fast or a slow buffer can block calcium elevation in the bulk of the spine head. The aforementioned experiments showed that CaMKII activation can be inhibited by either BAPTA or EGTA, implying that activation of this enzyme is dependent on an elevation of calcium in the bulk of the spine head[22]. However, a nanodomain process may also be required for such activation, as a bulk calcium signal, when produced by depolarization of voltage-dependent calcium channels, is not sufficient to activate CaMKII.

The actual calcium sensor for CaMKII activation is not the enzyme itself, but the small diffusible molecule calmodulin. A new view of calmodulin activation has been recently presented[32]. This view is based on measurements showing that the on-rate constant for calcium binding to calmodulin is significantly higher than it is for calcium binding to calbindin, the other major calcium buffer in pyramidal cells. Reconstitution experiments and computer simulations indicate that because of the high intracellular concentration of calmodulin (~100 μM) and its high on-rate constant for calcium binding, this protein can account for much of what has been termed the ‘fast calcium buffer’[27, 33]. From a signaling perspective, these conditions make sense: a substantial fraction of the calcium that enters the spine becomes bound to calmodulin, making it an excellent detector of calcium and an efficient activator of CaMKII subunits, which are present in spines at a concentration of ~100μM[34].

The ability of local activation of NMDARs to lead to local activation of CaMKII is non-trivial because calcium, calmodulin and CaMKII can all diffuse. For a small diffusible molecule, the diffusion length scale is of the order of 1μm per ms. A spine can be as close as 1μm to another, yet somehow LTP remains localized to the stimulated spine. A rather satisfactory biophysical explanation can now be given for how the synapse specificity of CaMKII activation is achieved (BOX 2). In brief, the narrow spine neck slows diffusion out of the spine, and the lifetime of the activated state of these molecules is short compared with the time that would be required for diffusion to carry the signal to other spines[35, 36].

Box 2

Biophysical basis of the synapse specificity of long-term potentiation

Calcium, calmodulin and CaMKII are all diffusible (albeit not freely), but the narrow spine neck that connects the spine head to the dendrite helps the compartmentalization of these molecules[35, 36]. In general, a signal will be localized to the spine if the decay rate of its activity is faster than the spine–neck diffusion coupling. Early experiments using two-

photon imaging of caged fluorescein showed that the spine–neck diffusion coupling time can be estimated as:

$$\tau = Vl/(Ds)$$

where V is the spine volume, l is the spine neck length, s is the cross sectional area of the spine neck, and D is the diffusion constant [153]. Assuming V~0.2 μm^3, l~1 μm and s~0.05$^2\pi$, τ can be calculated as ~0.1 s for small molecules, with D of the order of 100 μm/s^2. Thus, the active state of a signaling molecule will be localized to the spine if the lifetime of this state is <100 msec.

This condition is met by calcium. The spine–neck diffusion coupling of calcium (D~200 μm/s^2) is ~100 ms [27] and the clearance of calcium by extrusion or binding to a slow calcium buffer is ~20 ms [27], much faster than the spine–neck diffusion coupling. Thus, a calcium signal will stay largely within the activated spine.

The next step in the long-term potentiation cascade is the binding of calcium to the four binding sites on calmodulin. Calcium-saturated calmodulin keeps calcium bound for ~1 ms, a long enough time for calmodulin to diffuse within the spine and to bind CaMKII, but too short a period of time to diffuse out of the spine . Size considerations indicate that the upper bound for the calmodulin diffusion constant is similar to that of green fluorescent protein (~20 μm/s^2), yielding a diffusion coupling time of ~0.5 s. Thus, the decay time of activated calmodulin is considerably shorter than the time required for diffusion of calmodulin out of spines, ensuring localization.

The time constant for CaMKII inactivation is 45 s[22], but as CaMKII diffuses at a very slow rate (for CaMKII to exit spines may take many minutes), activated CaMKII is mostly localized to the activated spine.

Translocation and binding to the NMDA receptor

Once activated by calcium entry, CaMKII moves from the cytoplasm to the synapse, a phenomenon called translocation[37]. The drivers of this translocation are simple diffusion and the binding of activated CaMKII to synaptic proteins, most importantly NMDARs (see below). However, translocation is also due to spine enlargement, the resulting increase in F-actin in spines, and the resulting increase in CaMKII-beta/CaMKII-alpha heteromers bound to F-actin [38, 3940].

An important issue is whether translocation is synapse specific. This was demonstrated to be the case in experiments using two-photon glutamate uncaging to induce LTP at an identified spine (FIG. 1). A second method, using synaptically evoked LTP, showed similar results: in this case, active spines were identified by synaptically induced calcium elevation[41].

Electron microscopy immunolabeling[42] has been used to determine whether some of the CaMKII that translocates into the spine binds to the postsynaptic density (PSD). In this study, LTP induction, which was chemically induced, produced a two-fold increase in the amount of CaMKII per unit length of the PSD, as measured one hour after LTP induction

(FIG. 2). Other work has revealed the molecular processes that contribute to CaMKII binding to the PSD. Of note, activated CaMKII has been shown to bind NMDARs, most strongly to NR2B[43, 44]. Several binding sites involved in the formation of CaMKII–NMDAR complexes have been identified. The region near S1303 in NR2B binds activated CaMKII in a way that does not require T286 phosphorylation; a second binding site in NR2B between amino acids 839 and 1,120 does require T286 phosphorylation[45]. A I205K-encoding mutation in CaMKII, which affects the so-called T-site of the enzyme (BOX 1), blocks complex formation and strongly attenuates CaMKII translocation in HEK293 cells expressing CaMKII and NMDARs[46]. Activation of NMDARs in hippocampal slices leads to a two-fold increase in the amount of the CaMKII–NMDAR complex (FIG. 4D), as determined by coimmunoprecipitation experiments[43]. In experiments in which CaMKII translocation was caused by ionophore-mediated calcium entry, once CaMKII become bound to an NMDAR via the T-site, the complex persisted for more than 30 min after the removal of calcium[47]. Importantly, the binding of CaMKII to NR2B locks the enzyme in an active state[45] (but see [48]).

Formation of the CaMKII–NMDAR complex seems to have a key role in LTP induction and learning. LTP is greatly reduced by overexpression of mutant variants of NR2B (variants with mutations affecting residues near S1303) that block CaMKII binding to the NMDAR[49] (FIG. 2). The results of this study are in agreement with other experiments showing that total deletion of the cytoplasmic tail of NR2B can block LTP despite having no effect on NMDAR-mediated currents[50]. Transgenic mice have been generated that overexpress the cytoplasmic tail of NR2B, which interferes with the formation of the CaMKII–NMDAR complex and reduces the level of this complex by 20–50%[51]. These animals have a reduction in LTP of ~50% and exhibit learning deficits.

Although the NMDAR is probably of primary importance in initiating the binding of CaMKII to the PSD, other CaMKII-binding proteins may also have a role in this process. PSDs in the average spine contain about 80 CaMKII holoenzymes and the molar ratio of PSD-95 (a synaptic scaffolding protein) to NR1 is about 7:1[52, 53]. Thus, assuming that each NMDAR has two NR1 subunits, the PSD-95:NMDAR molar ratio is 14:1. The absolute number of PSD-95 molecules is ~300[52, 54], so a rough estimate of NMDARs per synapse is 20–25. Thus, many of the CaMKII holoenzymes may be held in the PSD by other binding partners, including densin-180 (also known as leucine-rich repeat-containing protein 7)[55], α-actinin[55], synapse-associated protein 97 (also known as disks large homologue 1)[56], and multiple PDZ domain protein[57]. Also, CaMKII is known to associate with other channels and receptors, including L-, T-, and P/Q-type voltage-gated calcium channels[58–60] and dopamine receptors[61]. Many of these channels and receptors are associated with the PSD and could therefore contribute to CaMKII binding. CaMKII holoenzymes can also bind to other holoenzymes in a self-association process[62]. Such binding serves to amplify the amount of CaMKII that is bound in the PSD and probably gives rise to the CaMKII towers that are observed in the PSD[63]. Although the CaMKII in the PSD probably has special importance in the control of synaptic strength (see below), most of the CaMKII in the spine is not in the PSD[34].

In summary, the available data point to the following model: activated CaMKII diffuses to the synapse and binds to NR2B, an event that is necessary for LTP (FIG. 3). Other binding partners may further enhance the amount of CaMKII in the PSD and spine head. Interestingly, the number of NR2B-containing NMDARs is independent of synapse size[53]. Thus, one possibility is that the amount of the CaMKII–NMDAR complex is graded; with increasing rounds of stimulation, NR2Bs eventually become saturated with CaMKII. This scenario could potentially explain why the stronger a synapse becomes, the less it can be potentiated[64]. Similarly, this scenario would explain why the higher the fraction of CaMKII that is bound in the spine, the fewer the number of CaMKII molecules that can be added to the spine by further LTP[42]. The enzymatic and structural processes by which CaMKII enhances AMPAR-mediated transmission are discussed below.

Potentiation of AMPA receptor function

The enhancement of AMPAR-mediated transmission during LTP occurs through two processes (FIG. 3). One process increases the average single channel conductance of AMPARs, which can be measured by non-stationary noise analysis[65]. A similar increase occurs after overexpression of an active truncated form of CaMKII[66]. The second process involves an increase in the number of AMPARs at the synapse[67, 68]. One of the studies revealing this process showed that LTP produced an increase in the rectification of the EPSC of the type expected from insertion into the membrane of AMPAR glutamate receptor 1 (GluR1) subunit homomers (GluR1 was overexpressed in this study). Further analysis revealed that extrasynaptic AMPARs that diffused into the synapse could be phosphorylated by CaMKII[68]. This phosphorylation occurs on the AMPAR auxiliary protein stargazin and allows the receptors to bind to PSD-95[69, 70]. In this way, AMPARs become captured by the synapse. We will now review the evidence for these processes.

GluR1 contains several important phosphorylation sites: S831, which is phosphorylated by PKC or CaMKII[71, 72]; S567, which is phosphorylated by CaMKII[73]; S845, which is phosphorylated by PKA[74]; and S818, which is a substrate for PKC[75]. Evidence now suggests that the increase in AMPAR conductance that is observed during the early stages of LTP is due to S831 phosphorylation. Of note, the phosphorylation of this site increases during LTP[76, 77]. Moreover, S831 phosphorylation blocks the increase in AMPAR conductance that is observed when active CaMKII is expressed (FIG. 4)[78]. Finally, a knockin mutation that encodes S831 pseudophosphorylated GluR1 increases channel conductance. Single-channel recordings show that S831 phosphorylation does not change the magnitude of any of the conductance states of AMPARs but, rather, enhances the probability that high conductance states will be activated by intermediate glutamate concentrations[78]. Interestingly, for AMPARs containing both GluR1 and GluR2, the ability of S831 phosphorylation to increase channel conductance only occurs if stargazin is present [78, 79]. Thus, understanding the interactions of subunits within the AMPAR is likely to provide mechanistic insight into channel regulation. Given that S831 phosphorylation occurs during LTP and that such phosphorylation increases the average channel conductance, one would expect that a knockin mutation encoding GluR1 S831A by itself would decrease the magnitude of LTP. Such an effect, however, is not observed unless

GluR1 S845 phosphorylation is also prevented[80]. Thus, the increase in AMPAR conductance during LTP may depend on interactions between the two sites.

The CaMKII-dependent phosphorylation of GluR1 probably occurs at the synapse itself. This scenario is suggested by the fact that extrasynaptic membrane patches that are excised after LTP induction have a higher number of AMPARs than do such patches prior to LTP induction (see below) but show no change in single-channel conductance[81]. The possibility that phosphorylation occurs at the synapse itself is further supported by the observation that activation of CaMKII in PSD preparations leads to strong phosphorylation of S831[82] (but see [83]).

As stated, the second mechanism leading to enhancement of AMPAR-mediated currents during the early phases of LTP is the insertion of AMPARs into the synapse. One step in this process could involve the exocytosis of AMPAR-containing vesicles into the plasma membrane, thereby supplying a pool of receptors that could be inserted into the synapse[84–86]. This step has been shown to occur by imaging of pHluorin-tagged AMPARs that only exhibit fluorescence when they are exposed to the high pH extracellular environment. After induction of LTP at single spines, a rapid increase occurs in the rate of exocytotic events, a rise that lasts for about a minute. These events occur in the stimulated spine and also in dendrites within 3 μm from the stimulated spine. Exocytosis depends on the RAS–extracellular signal-regulated kinase (ERK) pathway [86], RAB-GTPase proteins, soluble NSF attachment protein receptors (SNARE proteins), syntaxin 4 and 13, and the motor protein myosin V. However, inhibition of any of these proteins does not affect the first 20 minutes of LTP, although it does strongly reduce potentiation at later times (Fig. 4)[87–94]. The fact that inhibition of exocytosis does not affect the first 20 minutes of potentiation suggests that this early phase is due to the capture of AMPARs that are present extrasynaptically before LTP induction[85].

The calcium sensor that triggers the exocytosis pathway is unclear. The RAS– ERK pathway can be stimulated by CaMKII[94], but exocytosis is only partially blocked by the CaMK inhibitor KN62[86]. This finding raises the possibility that there are other calcium sensors for exocytosis, perhaps one of the calcium-sensitive RAS activators (guanine nucleotide exchange factors or GEFs) or inactivators (GTPase-accelerating proteins or GAPs)[95].

Insertion of AMPARs into the extrasynaptic membrane is not sufficient to enhance transmission; translocation of these channels into the synapse requires the phosphorylation of stargazin, probably by CaMKII[69, 83]. This requirement was demonstrated by mutating the putative CaMKII phosphorylation sites of stargazin to preclude phosphorylation. Although these mutations did not affect the surface expression of AMPARs, they did prevent these channels from enhancing synaptic transmission after LTP induction (Fig. 4). Conversely, pseudophosphorylation of stargazin led to enhanced transmission that occluded LTP. These findings suggest that LTP-induced phosphorylation of stargazin is required for the capture of extrasynaptic AMPARs by the synapse.

This capturing process has now been directly observed using quantum-dot labeling of AMPARs and single-particle tracking[70]. Under basal conditions, AMPARs diffused freely

in the extrasynaptic space while pausing only briefly at synaptic locations. Upon activation of CaMKII by NMDA application or synaptic input, diffusing AMPARs were captured at synapses (Fig. 4). The arrest of AMPARs at stimulated synapses is dependent on the phosphorylation of stargazin, as receptors with non-phosphorylatable stargazin did not exhibit immobilization at synapses. Immobilization of AMPARs could also be blocked by inhibition of CaMKII. Thus, although stargazin can be phosphorylated by either CaMKII or PKC, CaMKII seems to have the dominant role in stimulating the capture process. Capture was also prevented by expressing a mutant variant of CaMKII that is unable to bind NMDARs but still allows CaMKII activation. Given that NR2B is at the synapse, these results suggest that the stargazin phosphorylation that triggers AMPAR capture occurs at the synapse rather than extrasynaptically.

It was initially thought that AMPARs were bound in the synapse through the association of GluR1 subunits with PSD proteins having PDZ, but this seems now not to be the case[96]. Rather, the synaptic localization of AMPARs depends on the binding of stargazin to PSD-95[97, 98]. Synaptic clustering of AMPARs can be inhibited by mutating the binding sites on stargazin that bind to PSD-95 or by mutating the binding site on PSD-95 that binds stargazin [98]. As noted earlier, the early phase of LTP can be blocked by deleting the CaMKII phosphorylation sites on stargazin. If the role of this phosphorylation is to allow stargazin to bind to PSD-95, then early LTP should also be blocked by deleting the PDZ-1 and PDZ-2 domainson PSD-95 to which stargazin binds. Consistent with this prediction, overexpression of PSD-95 lacking these domains prevents LTP induction[99].

Additional evidence for the importance of the stargazin–PSD-95 interaction comes from the study of basal transmission in CA1[100]. Application of a ligand that disrupts both PDZ-1 and PDZ-2 interactions produced a rapid reduction in EPSCs and miniature EPSCs (mEPSCs). The fact that the saturating concentration of the ligand produced only a 45% reduction in these currents indicates that only a component of AMPAR-mediated transmission is dependent on the stargazin–PSD-95 interaction. This notion is consistent with previous work showing that mutliple mechanisms underlie basal AMPAR-mediated transmission at CA1 synapses[101, 102].

A structural picture for how phosphorylation of stargazin leads to anchoring of receptors at the synapse is beginning to emerge. The PDZ-binding site on stargazin is normally situated close to the membrane, where it can be held by electrostatic interactions with positively charged lipids[103]. Synaptic PSD-95 has been visualized by electron microscope tomography and is oriented perpendicular to the membrane with the PDZ-1 and -2 domains that bind stargazin situated quite far (15 nm) from the membrane [104]. CaMKII phosphorylation of stargazin may interfere with the electrostatic interactions at the membrane, allowing the PDZ ligand sites on stargazin to extend from the membrane and bind to PSD-95.

Thus, there is now rather strong support for a relatively simple model of early LTP (FIG. 3). The process begins with CaMKII activation. CaMKII then binds to the NMDAR, putting the kinase in close juxtaposition to GluR1 and to the carboxy-terminal region of stargazin[83]. The resulting phosphorylation of GluR1 enhances AMPAR conductance, while the resulting phosphorylation of stargazin leads to the anchoring of additional AMPARs at the synapse.

Together, these processes enhance synaptic transmission by an enzymatic process. The duration of this enzymatic action may be only several minutes[105], but the consequences of CaMKII enzymatic activity may last longer because it takes time for the substrates to become dephosphorylated.

Maintenance of long-term potentiation

We now turn our attention to the later phases of LTP and the processes that maintain synaptic strength. Less is known about these processes and the specific role of CaMKII. Most of the CaMKII in spines is active only for about a minute after synaptic stimulation[22], suggesting that CaMKII may exert its enzymatic effects for only this brief period. Thus, one view is that the involvement of CaMKII in LTP is only transient and that different downstream processes are required to maintain LTP[106]. Support exists, however, for an alternative scenario in which a small subset of CaMKII molecules has a persistent role in LTP, a role that may be structural rather than catalytic. Several lines of evidence point to a long-lasting role. As mentioned above, formation of CaMKII–NMDAR complexes is required for LTP induction[49] (Fig. 2). In a neuronal cell-culture model of LTP, these complexes, once formed, can be persistent[47]. Furthermore, the LTP-induced association of CaMKII with the PSD can persist for at least 60 min after induction (Fig. 2). Finally, large pools of CaMKII–NMDAR complexes exist in the hippocampus under basal conditions[43], perhaps resulting from previous learning (Fig. 2). Thus, one hypothesis is that synaptic strength is controlled by the amount of CaMKII–NMDAR complex that is present in the synapse.

A key test of any model of synaptic memory mechanisms is to inhibit or disrupt the putative memory molecule after LTP induction and show that saturated LTP can be reversed. However, this result might simply indicate interference with LTP expression, i.e. processes that couple the memory mechanism to the change in AMPAR properties. To show that a maintenance mechanism has been affected, two further tests are required. First, the attacking molecule must be subsequently removed; if an expression process was affected, LTP will recover, whereas if a maintenance process was affected, LTP will not recover. Second, to exclude the possibility that the manipulations have damaged a cell, it must be shown that LTP can be re-induced.

Tests of this kind have been conducted to test the role of the CaMKII–NMDAR complexe in the maintenance of LTP. These tests used CN compounds, a class of CaMKII inhibitor known to interfere with the binding of CaMKII to NR2B *in vitro.* As shown in (Fig. 2), transient treatment of a hippocampal slice with CN compound reversed LTP, allowing additional LTP to be induced[107]. In parallel experiments, it was shown that CN inhibitor reduced the amount of the CaMKII–NMDAR complex in the slice.

A further conclusion of these experiments is that CN inhibitors reverse LTP by interfering with the binding reactions of CaMKII rather than catalytic actions of the kinase. CN compounds bind strongly to the T-site on CaMKII to which the NMDAR binds[108] whereas other CaMKII inhibitors bind mainly to the catalytic site of the enzyme (the S-site) and only weakly to the T-site. The latter type of inhibitor does not reverse LTP[109, 110]. These findings

also explain why low concentrations of CN compounds that are sufficient to block CaMKII activity[111], but are not sufficient to disrupt the CaMKII–NMDAR complex, do not reverse LTP[107]. These results taken together, suggest that in addition to the catalytic action of CaMKII important for synaptic strengthening during early LTP, there is a structural process by which CaMKII strengthens transmission during late LTP.

Not much can be said with certainty about what this structural process might be, but there are several lines of relevant evidence. LTP is accompanied by a rapid and persistent increase in spine volume (Fig. 1), an increase that can be mimicked by overexpression of active CaMKII holoenzymes[11]. In addition to the increase in spine volume, there appears to be a slow increase in the size of the synapse itself (both pre- and postsynaptically)[112]. The late phase of spine growth is dependent on protein synthesis[113], which is stimulated by brain-derived growth factor and dopamine and involves the selective targeting of newly synthesized proteins to the synapses that have undergone LTP[114–116]. Changes in protein synthesis are due in part to local control mechanisms[117] and in part to transcriptional changes[118]. Changes in DNA methylation control some of the long-term transcriptional changes[119]. Ultimately, there is change in the synthesis of many proteins and these include CaMKII. A mutation in CaMKII that specifically interferes with its dendritic synthesis blocks late LTP and greatly reduces the CaMKII content of the PSD[120].

Another perspective on the structural changes in late LTP comes from consideration of the changes in quantal transmission. According to one class of models[122, 123], the synapse is a modular structure, with each module capable of anchoring ~20 AMPARs (the total number of AMPARs at the synapse can be in the hundreds). During LTP, the addition of AMPARs causes these modules to become functional while coordinated presynaptic processes increase the size of the active zone and enhance the full-fusion mode of vesicle release. Thus, in the late phase of LTP, a synapse is stronger both because of enhanced glutamate release and because there are additional AMPAR-containing postsynaptic modules. The mechanisms by which these structural changes are triggered and the role of CaMKII in these processes is not known.

Conclusions and perspectives

Although there has been progress in understanding LTP, many important questions remain unanswered. The ability to optically measure LTP-induced calcium elevation and CaMKII activation in single spines raises the prospect of understanding the initial processes of LTP at a quantitative level. Models of increasing sophistication[121, 122] have been developed for how CaMKII is activated by calcium and calmodulin *in vitro*. Using these models to understand how CaMKII is activated in the living cell requires knowledge of the state of calmodulin, about which less is known. The abundant protein neurogranin binds calmodulin and releases it when Ca^{2+} binds to calmodulin. Mutations in the neurogranin gene have strong effects on synaptic plasticity[123], some of which can be accounted for by computational models[124]. Direct measurements of the state of free and bound calmodulin will be important in providing the information necessary to quantitatively accounting for CaMKII activation.

The focus of this Review has been on T286 phosphorylation, but there are other phosphorylation sites on CaMKII about which less is known. A short time after T286 phosphorylation, T305 and T306 in the calmodulin-binding region become phosphorylated[125]. This delayed phosphorylation strongly reduces autonomous kinase activity[126, 127]. The phosphorylation of T305 and T306 has physiological consequences: overexpression of T286D CaMKII (to mimic the phosphorylated state) actually leads to synaptic weakening (and occlusion with long-term depression (LTD)) unless T305 and T306 phosphorylation is prevented by additional mutations[128]. Phosphorylation of these sites prevents binding of CaMKII to the PSD and produces learning deficits[129]. Understanding the conditions that lead to T305 and T306 phosphorylation will have medical implications because Angleman's disease, a form of intellectual disability, is associated with enhanced phosphorylation at these sites that prevents normal LTP[130]. CaMKII contains other phosphorylation sites, for example T253, the functional role of which is unclear[131, 132]. Recent work shows that stimuli that induce moderate levels of calcium elevation cause translocation of CaMKII to inhibitory synapses, leading to enhancement of $GABA_A$ receptor insertion[133]. It will be interesting to determine the properties of CaMKII that target this protein to inhibitory versus excitatory synapses.

There has been substantial progress in understanding the role of CaMKII in LTP expression, but there remain uncertainties. Experiments suggest that the different expression processes (that is, enhancement of channel conductance versus an increase in channel number) may dominate at different synapses on the same cell[65]. Why this should be is completely unclear. The role of different GluR1 phosphorylation sites and their interactions remain unclear, particularly the role of CaMKII-dependent phosphorylation of S567. Aspects of the role of stargazin in LTP also remain uncertain. It is likely that stargazin phosphorylation is a required step for transition of AMPA receptors from the plasma membrane to the synapse, but the kinetics of this phosphorylation step are not known. Furthermore, if the binding of stargazin to PSD-95 leads to AMPAR-mediated transmission, then the effects on LTP of deleting the PDZ l binding domain of stargazin should be equivalent to the expression of a nonphosphorylatable mutant of this protein, but they are dramatically different, suggesting that the stargazin-mediated mechanism involves additional complexity.

The mechanisms of LTP maintenance remain controversial. Evidence has been presented that protein kinase M ζ (PKM-ζ) is responsible for the maintenance of LTP. Moreover, various forms of memory, as measured at the behavioural level, can be erased by PKM-ζ inhibitors[134, 135]. However, questions remain regarding the specificity of ZIP[115, 136], the main pharmacological agent used to inhibit PKM-ζ, and it will be important to test the PKM-ζ hypothesis of LTP maintenance by knockout methods. A preliminary report indicates that late LTP is not affected by knocking out PKM-ζ expression and that the effects of ZIP still occur in knockout animals[137].

An alternative to the PKM-ζ hypothesis is that LTP is maintained by the CaMKII–NMDAR complex. This model is supported by the ability of CN compounds to reverse LTP maintenance[107] (Fig. 2). Although it is known that formation of this complex is necessary for LTP and that the complex forms during chemical forms of LTP, methods need to be developed to monitor CaMKII–NMDAR complexes during synaptically-induced LTP. Such

methods would make it possible to determine whether the complexes that form have the stability to underlie long-term memory. The fraction of total spine CaMKII subunits that are bound to the NMDAR is probably too small (< 1%)[34] to be monitored by optical indicators of CaMKII activity, so the monitoring of such complexes will thus require more specific methods, such as those based on FRET between CaMKII and the NMDAR.

One of the most fundamental problems regarding information storage by synapses is how stable storage can be achieved despite the constant turnover of molecules at the synapse. Whatever the underlying mechanism, they must yield information storage that is stable over many years. One model attributes long-term storage to phosphorylated CaMKII in the PSD and suggests a specific mechanism whereby this phosphorylated state is maintained when unphosphorylated CaMKII proteins replace older phosphorylated ones during protein turnover[138]. According to this model, the high density of phosphorylated CaMKII molecules in the PSD saturates the level of phosphatase activity in the PSD; thus, a newly inserted CaMKII will become spontaneously phosphorylated because there is a basal rate of calcium-dependent autophosphorylation that is not effectively counteracted by the saturated phosphatases. Models of this kind seem to contradict the evidence for a structural rather than catalytic role of CaMKII in LTP maintenance. That evidence shows that peptides that interfere with the CaMKII–NMDAR complex reverse LTP maintenance within 30 min, whereas peptides whose action is primarily at the catalytic site do not. However, these data do not exclude the possibility that maintenance, in addition to depending on a structural readout of the CaMKII–NMDAR complex, requires CaMKII-dependent autophosphoryhlation over a longer time scale. Indeed, biochemical work strongly hints that keeping PSD CaMKII phosphosphorylated is important. This work shows that although protein phosphatase-1 at the PSD dephosphorylates some sites on CaMKII, it cannot dephosphorylate T286[139]. Recent work shows that T286 sites cannot be dephosphorylated because the NMDAR itself shields these sites[140]. On the other hand, there are also hints that long-term memory storage may not depend on autophosphorylation of T286. Whereas long-term memory for one-trial learning is strongly attenuated in T286A CaMKIIα mice, memory after massed training persists[141], suggesting that synapses have other mechanisms for memory storage. Whether this is due to very different molecules or to CaMKII-β activity remains unclear[142].

Development of an understanding of how informational stability can be achieved during protein turnover will require information about how synaptic proteins are actually synthesized and degraded. Experiments indicate that CaMKII is involved in stimulating both protein synthesis and ubiquitin-dependent protein degradation [143–145]. Further work will be required to understand these slow reactions and the mechanisms by which the stability of stored information is ensured.

In summary, our knowledge about LTP is advancing, but major questions remain. The greatest clarity is about the earliest steps involving activation of the NMDAR, the accumulation of calcium, and the resulting activation of calmodulin and CaMKII. Furthermore, there is impressive evidence indicating that early LTP results from CaMKII-dependent phosphorylation of GluR1 and stargazin. Further work will be required before this mechanism can be considered definitive, however. The late stages of LTP, the stages

most important for understanding long-term memory, remain much more mysterious. The late stages are clearly more complex than early ones because they involve changes in gene expression, protein synthesis, and synapse structure and number.

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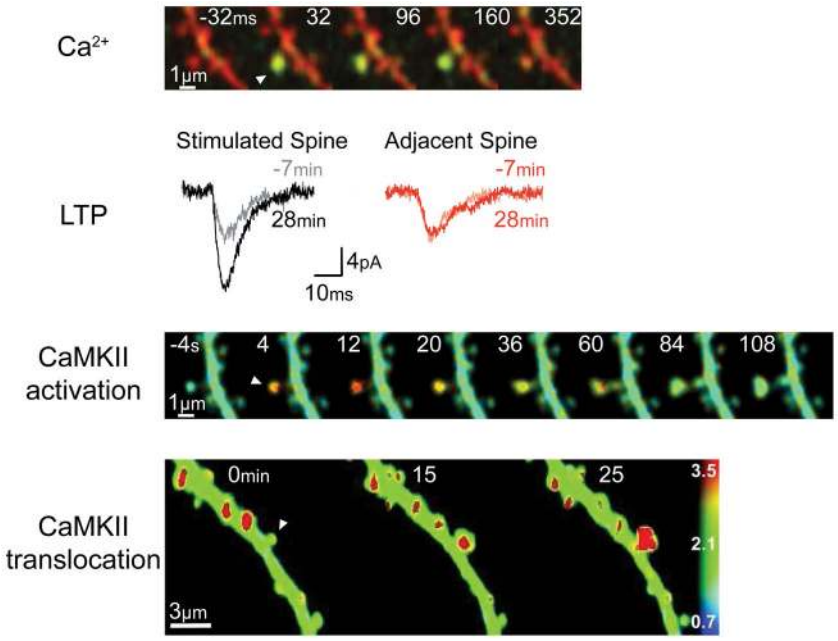

Figure 1. Synapse-specificity of the processes involved in LTP induction

a A transient elevation of calcium (detected by the calcium-sensitive dye Fluo4-FF; green signal) in a spine can be observed in response to glutamate uncaging at that spine (arrowhead). The calcium-insensitive dye Alexa-594 outlines the spine volume (red). [35]. **b** Local glutamate uncaging can potentiate AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) in the stimulated spine but not in an adjacent spine[154]. **c** After LTP induction by local glutamate uncaging (arrowhead), transient activation of CaMKII can be observed (red denotes high activation; level of activation measured by the FRET sensor Camui). [22]. **d** Stimulation of a spine (arrowhead) in this fashion can cause a persistent increase in spine volume and translocation of CaMKII (here shown by translocation of green fluorescent protein-labelled αCaMKII; red denotes high levels of this kinase)… From [41].

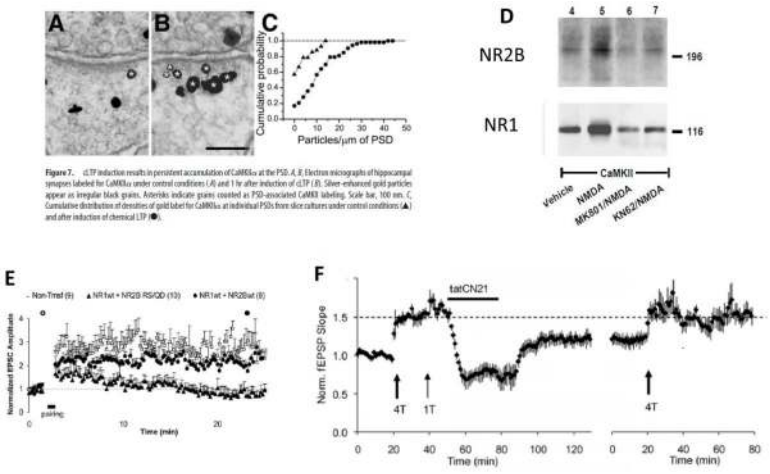

Figure 2. Role of CaMKII–NMDAR complex in LTP maintenance

Electron microscopy immunolabelling has been used to measure CaMKII levels in postsynaptic densities (PSDs) **a** before and **b** one hour after chemically induced LTP. **c** The histogram shows that there is more CaMKII per unit length of the PSD after LTP induction than before induction of LTD [42]. **d** The CaMKII–NMDAR complex can be immunoprecipitated with an antibody that detects CaMKII. Synaptic stimulation with NMDA increases the amount of NR2B and NR1 in complexes with CaMKII. These increases can be blocked by the NMDAR antagonist MK801 or by the CaMK inhibitor KN62 [43]. **e** Overexpression of a mutant form of NR2B (RS/QD) blocks LTP (LTP induction denoted by the arrow) [49]. **f** LTP (induced at arrow) can be reversed by tatCN21, a peptide that interferes with CaMKII binding to NR2B. In this example, LTP was induced by 4 tetani (T), and an additional tetanus had no effect, indicating that LTP saturation had been reached (left-hand side). Subsequent application of tatCN21 reversed LTP. After 30 mins of tatCN21 application, the peptide was removed and only partial recovery of LTP was observed (note that there was an acute inhibitory effect that was reversed when the peptide was removed). Additional LTP could then be induced using a 4T protocol (right-hand panel). [107]

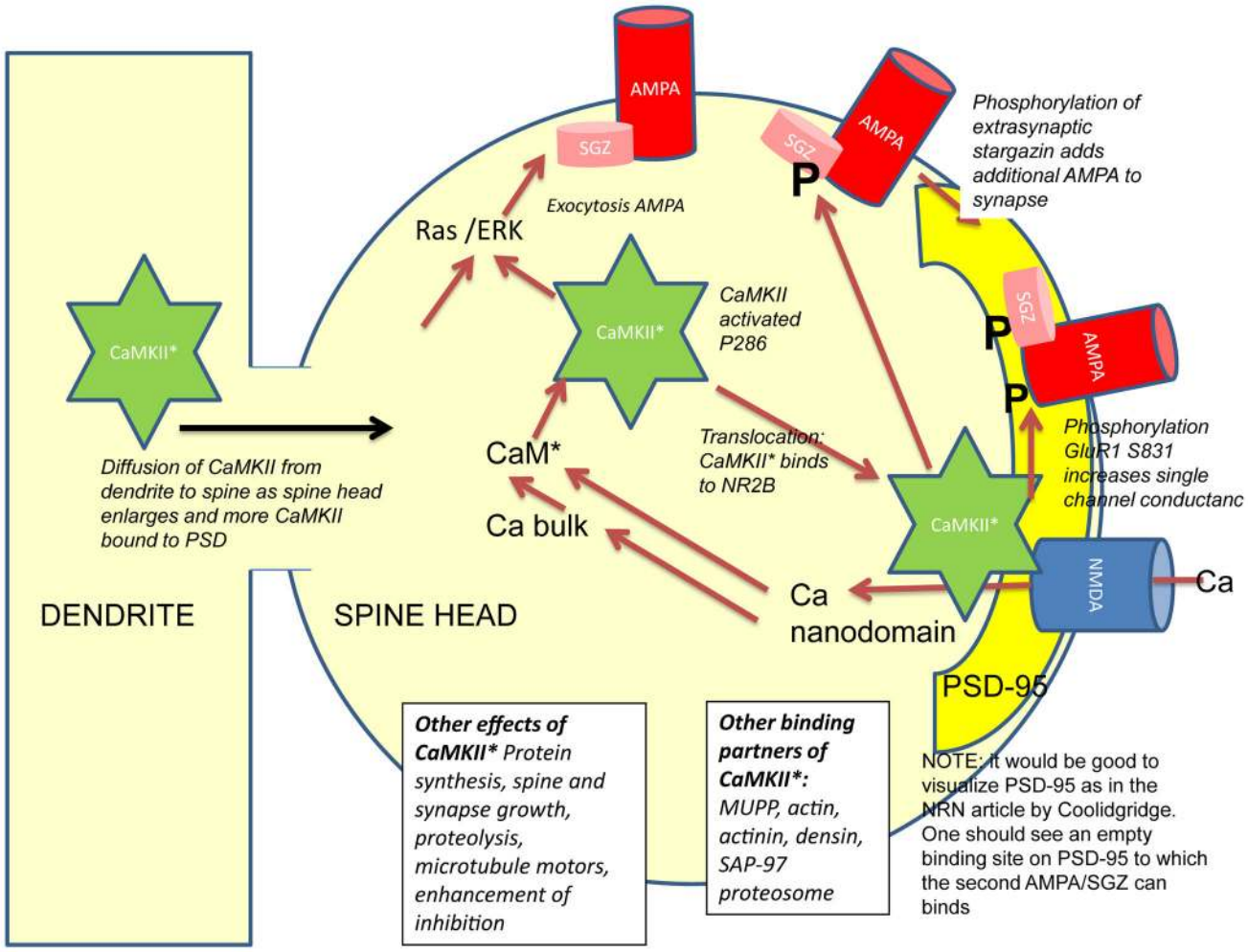

Figure 3. Role of CaMKII is in AMPA receptor-mediated transmission during early LTP

Calcium entry through the NMDAR activates calmodulin both in the bulk cytoplasm and in the channel nanodomain. Calmodulin activates CaMKII, which then translocates to the PSD where it enhances AMPAR-mediated transmission in two ways: 1) by phosphorylation of GluR1 and S831, which leads to increased average conductance of the channel and 2) phosphorylation of stargazin, which leads to its binding to PSD-95, thereby increasing the number of AMPAR at the synapse. CaMKII, perhaps with other calcium dependent processes, stimulates fusion of vesicles containing AMPAR with the plasma membrane, increasing the extrasynaptic channel concentration. This increase is not necessary for the earliest stage of LTP, but is necessary for subsequent stages.

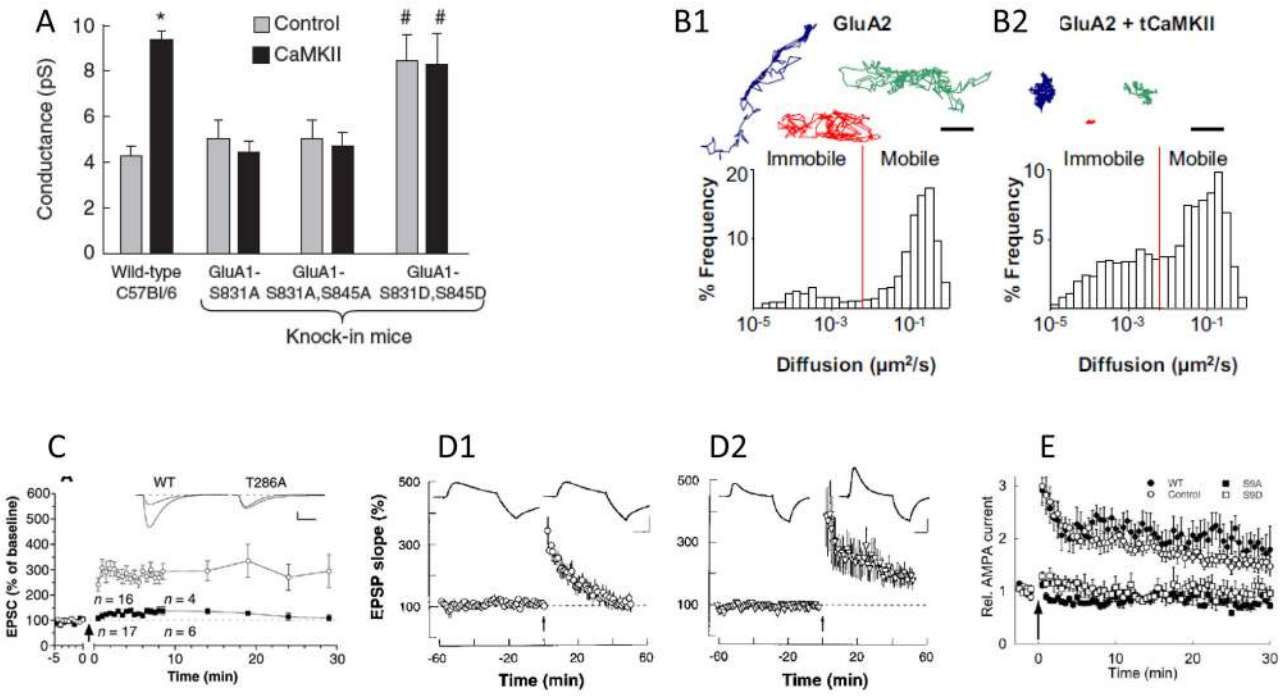

Figure 4. Mechanisms of expression processes by which CaMKII enhances AMPA receptor-mediated transmission

a CaMKII enhances AMPA receptor (AMPAR) conductance in isolated CA1 pyramidal cells of wild-type mice but not in knockin mice in which phosphorylation of the AMPAR subunit glutamate receptor 1 (GluR1) on S831 or both S831 and S845 is prevented by mutation of the serine residues to alanine residues. Pseudophosphorylation of these sites (that is, expression of S831D or S845D GluR1) enhances AMPAR conductance and prevents further enhancement by CaMKII [78]. **b** In addition to affecting AMPAR conductance, CaMKII also affects the mobility of these receptors. Single-particle tracking experiments have shown that in neurons under control conditions (left-hand panel), GluR2 subunits exhibit rapid diffusion that follows a broad path (three examples of particle paths are shown at the top and a histogram of diffusion constants for GluR2-labelled particles is shown at the bottom). In neurons overexpressing an active (truncated) form of CaMKII (tCaMKII; right-hand panel), GluR2 molecules show limited diffusion paths (top) and a lower average diffusion constant (bottom), as AMPARs are captured at the synapse[70] **c** The influence of CaMKII on AMPA receptor-mediated transmission can be observed in LTP experiments. LTP can be induced by a pairing protocol (arrow denotes the point of protocol initiation) in wild-type CA1 pyramidal cells but is prevented in cells expressing a mutant variant of CaMKII (T286A) that cannot become persistently active [12]. **d Block of exocytosis of AMPAR containing vesicles** by postsynaptic injection of botulinum toxin (left-hand panel) did not affect earliest phase of LTP; control is shown after injection of inactivated botulinum toxin (right-hand panel) [91]**. e** Stargazin has an important role in CaMKII-enhanced AMPA receptor-mediated transmission. LTP is blocked in CA1 pyramidal cells expressing a mutant variant of stargazin in which the nine potential CaMKII/PKC serine phosphorylation sites are mutated to alanine residues (S9A). LTP can be induced in cells

expressing pseudophosphorylated stargazing (S9D). The control condition is without LTP induction, and the arrow denotes the start of the induction protocol [69].