



REVIEW

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# The CaMKII/NMDAR complex as a molecular memory

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## Abstract

CaMKII is a major synaptic protein that is activated during the induction of long-term potentiation (LTP) by the  $\text{Ca}^{2+}$  influx through NMDARs. This activation is required for LTP induction, but the role of the kinase in the maintenance of LTP is less clear. Elucidating the mechanisms of maintenance may provide insights into the molecular processes that underlie the stability of stored memories. In this brief review, we will outline the criteria for evaluating an LTP maintenance mechanism. The specific hypothesis evaluated is that LTP is maintained by the complex of activated CaMKII with the NMDAR. The evidence in support of this hypothesis is substantial, but further experiments are required, notably to determine the time course and persistence of complex after LTP induction. Additional work is also required to elucidate how the CaMKII/NMDAR complex produces the structural growth of the synapse that underlies late LTP. It has been proposed by Frey and Morris that late LTP involves the setting of a molecular tag during LTP induction, which subsequently allows the activated synapse to capture the proteins responsible for late LTP. However, the molecular processes by which this leads to the structural growth that underlies late LTP are completely unclear. Based on known binding reactions, we suggest the first molecularly specific version of tag/capture hypothesis: that the CaMKII/NMDAR complex, once formed, serves as a tag, which then leads to a binding cascade involving densin, delta-catenin, and N-cadherin (some of which are newly synthesized). Delta-catenin binds AMPA-binding protein (ABP), leading to the LTP-induced increase in AMPA channel content. The addition of postsynaptic N-cadherin, and the complementary increase on the presynaptic side, leads to a trans-synaptically coordinated increase in synapse size (and more release sites). It is suggested that synaptic strength is stored stably through the combined actions of the CaMKII/NMDAR complex and N-cadherin dimers. These N-cadherin pairs have redundant storage that could provide informational stability in a manner analogous to the base-pairing in DNA.

CaMKII is a highly abundant brain protein concentrated in the postsynaptic density (PSD) and is strongly implicated in LTP (reviewed in [1]). During the induction of LTP,  $\text{Ca}^{2+}$  enters through the NMDAR and binds to calmodulin [2]. Calmodulin then activates CaMKII, which phosphorylates the GluA1 subunits of AMPARs and an auxiliary subunit of AMPARs, stargazin. The first reaction increases the conductance of AMPARs [3,4]; the second allows more AMPARs to be bound into the synapse by PSD-95 [5,6]. Together, these processes provide a mechanistic explanation for the early phase of LTP (approximately the first 30–60 minutes). Later phases of LTP appear to require different mechanisms, and it is these mechanisms that maintain LTP for the long periods

required for memory storage. The hypothesis that we will evaluate in this review is that late LTP is maintained by the complex of CaMKII with the NMDAR. Evidence relevant to the following criteria will be summarized:

1. LTP induction should cause a persistent increase in the CaMKII/NMDAR complex.
2. Inhibiting formation of the CaMKII/NMDAR complex should block LTP induction.
3. Decreasing the amount of CaMKII/NMDAR complex after LTP induction should reverse LTP.
4. A component of *basal* transmission should be reversed by reducing the basal CaMKII/NMDAR complex.
5. There must be a mechanism by which CaMKII/NMDAR complex can produce potentiation, specifically the trans-synaptic structural processes that underlie late LTP.

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### **LTP induction should cause a persistent increase in the CaMKII/NMDAR complex**

The initial evidence that CaMKII could interact with the NMDAR came from *in vitro* experiments showing that a fragment of GluN2B is a substrate for purified CaMKII (at 1303) [7]. It was noted that the binding affinity of GluN2B was much higher than for other CaMKII substrates. Subsequent experiments showed that CaMKII could form a tight complex with NMDAR and that the complex was present in living cells, as identified by crosslinking or coimmunoprecipitation (co-IP) [8-10]. Importantly, the amount of complex was increased substantially by stimulation that elevated intracellular  $Ca^{2+}$  and thereby activated CaMKII.

A critical assumption of the proposed model is that LTP produces an increase in the CaMKII/NMDAR complex that is persistent for the duration of LTP. This assumption remains to be tested directly, but there is relevant evidence. Stimulation of cultured neurons with glutamate/glycine (a form of chemical LTP) can trigger persistent translocation of CaMKII that requires kinase binding to GluN2B at what is termed the T-site [11]. Importantly, the persistently translocated CaMKII is phosphorylated at T286 [12]. Furthermore, chemical LTP produces translocation of CaMKII to the PSD, as visualized by electron microscopy; this persists for at least 1 hr after LTP induction, suggesting that there is persistent formation of the CaMKII/NMDAR complex [13,14].

The binding of CaMKII to the NMDAR requires an activated open form of the kinase. Therefore, the recent optical experiments [15] demonstrating that CaMKII is only transiently activated after LTP induction (~1 minute) could suggest that the CaMKII/NMDAR complex is also transient. However, there are two reasons to doubt the generality of this conclusion. First, recent measurements indicate that the fraction of CaMKII subunits directly bound to the NMDAR and thereby locked in the open confirmation [16] is a small fraction of the total CaMKII in spines and may be difficult to detect optically [17]. Second, the optical measurements of CaMKII activation were made under conditions that did not evoke late LTP [18], raising the possibility that future measurement under conditions that did induce late LTP would evoke a detectable persistent component, as seen with induction protocols based on tetanic stimulation [19]. In any case, given that the key question is the duration of the LTP-induced increase in CaMKII/NMDAR complex, what is needed is a method (possibly based on FRET) that would make it possible to directly monitor the kinetics of complex formation and persistence.

### **Inhibiting formation of the CaMKII/NMDAR complex should block LTP induction**

To examine the functional role of complex formation, Barria and Malinow [20] overexpressed a form of GluN2B having mutations near the CaMKII phosphorylation site (R1300Q and S1303D) that strongly interferes with binding [21]. LTP was examined using a low-frequency pairing protocol. There was a significant (but not complete) block of potentiation in the first 10 minutes after LTP induction; at later times, the block was complete.

Related experiments were conducted in hippocampal slices from knockin (KI) mice in which CaMKII-GluN2B binding was impaired by the mutations L1298A and R1300Q [22]. In these mice, the basal CaMKII/NMDAR complex was reduced by about 40%. Activity-dependent formation of complex in cell cultures was more strongly reduced. LTP induced by high-frequency stimulation in slice experiments was reduced, but only by about 50%. It is unclear whether the smaller reduction than in the Barria/Malinow experiments is due to a slightly different mutation, to the difference in animal age, or to a difference in the LTP induction protocol.

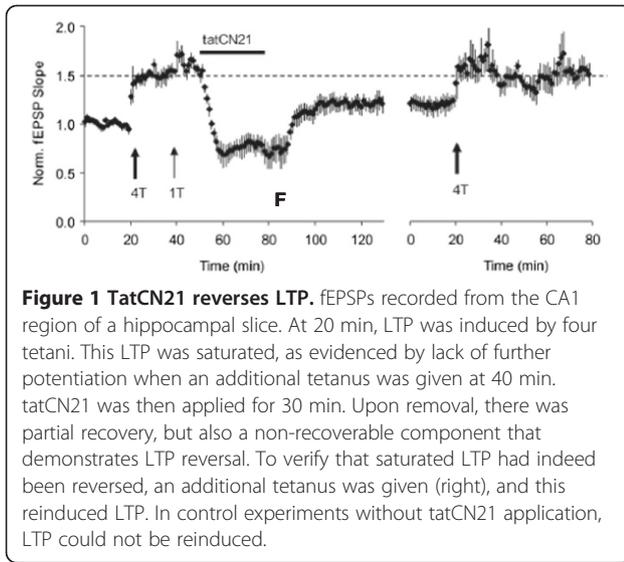
Another method for interfering with the CaMKII/NMDAR complex utilized transgenic mice in which the whole C-terminal GluN2B fragment could be induced [23]. The LTP induced by high-frequency stimulation was reduced by about 50%. It is unclear whether this reduction was due to inhibiting CaMKII activity or to inhibiting the formation of the CaMKII/NMDAR complex.

A complementary experiment would be to determine the effects of increasing the CaMKII/NMDAR complex. Overexpression of activated CaMKII holoenzyme increases synaptic strength and spine size, provided that phosphorylation of T305/306 is prevented [24]. It would be expected that this form of holoenzyme would increase the CaMKII/NMDAR complex and synapse size, but this has not been directly tested.

### **Decreasing the amount of CaMKII/NMDAR complex after LTP induction should reverse LTP**

An important tool has been the development of a related group of peptides (CN27; CN21; CN19) that block the formation of the CaMKII/NMDAR complex *in vitro* [25]. This peptide family derives from a fragment of an endogenous CaMKII inhibitor protein, CaMKIIN [26]. This protein strongly binds to a site on CaMKII (the T-site) in addition to the catalytic site to which other peptide inhibitors mainly bind (these are based on the sequence of the CaMKII regulatory domain). It is to the T-site that the NMDAR itself binds.

Application of CN21 can reverse LTP in hippocampal slices [27], as shown in Figure 1. An action of this kind would be consistent with an effect on either LTP expression or maintenance processes. An experiment that



distinguishes between these possibilities is to remove the peptide; if the effect was on expression, LTP should recover. Figure 1 shows that LTP does not return. It could be argued that this lack of return resulted from damage; however, the ability to then reinduce LTP argues against this possibility. Thus, CN21 appears to reset a molecular switch that controls LTP maintenance.

Several controls point to the fact that CN21 works in slices to reduce the CaMKII/NMDAR complex, as expected from *in vitro* work. First, the peptide reduced the amount of basal complex, as measured by co-IP (basal transmission was also reduced; see next section). If somewhat lower concentrations of CN21 were used (5  $\mu$ M instead of 20  $\mu$ M), there was no reduction in the CaMKII/NMDAR complex and no persistent reduction in basal transmission. Second, the peptide produced a small but statistically significant reduction in the amount of CaMKII bound in spines. Third, CN21 produced a large reduction in the activity-dependent increase of CaMKII in the PSD [28].

The ability of CN compounds to rapidly reverse LTP is not shared by other peptides that primarily inhibit the CaMKII catalytic site [29,30]. This suggests that the reversal is due to interference with structural rather than enzymatic reactions. This could mean that there is no role for enzymatic activity (including autophosphorylation-induced autonomous activity [16]) during LTP maintenance. Alternatively, the effects of inhibiting such activity may require many hours to develop and thus have not been seen in the much shorter experiments performed thus far. Indeed, experiments have shown that although phosphatase is present in the PSD, there is negligible dephosphorylation of CaMKII T286 within 1 hr [31].

### **A component of basal transmission should be reversed by reducing the basal CaMKII/NMDAR complex**

LTP has been observed *in vivo* during learning [32,33]. Moreover, synapses that have undergone learning-dependent LTP cannot then undergo LTP by electrical stimulation. This occlusion suggests that learning has saturated the normal LTP process. It would thus be expected that at least part of basal transmission, as measured in hippocampal slices, is the result of LTP-like events that occurred while the animal was alive. This inference is confirmed by analysis of unitary responses in the slice: the stronger the basal transmission, the smaller the magnitude of the LTP that can be induced [34,35].

Based on the data outline in the above paragraph, a strong expectation is that an agent that affects a synaptic memory mechanism should reduce basal transmission. Consistent with this, CN compounds depress basal transmission [27]. Moreover, this depression persists after removal of the CN compound, and the LTP that can then be induced is larger than if a scrambled control peptide or no peptide had been applied.

CN-induced depression is observed for drug concentrations necessary to disrupt basal CaMKII-NMDAR interaction in the slices, but not for lower concentrations causing only kinase inhibition [27]. Moreover, this type of depression is different from known forms of activity-dependent long-term depression (LTD), and it does not require  $Ca^{2+}$  influx, protein synthesis, or degradation [36]. The CN-induced persistent depression was barely detected in very young animals (P7-P10; [36]) for which CaMKII content at synapses is comparatively much lower [37,38]. Taken together, these findings strongly suggest that CN-induced depression of basal transmission is caused by breakdown of the CaMKII-NMDAR interaction at the synapse. Perhaps inconsistent with this conclusion is the fact that basal transmission was not reduced when the basal level of CaMKII/NMDAR complex was reduced by knockin of NMDAR mutations [22]. However, it is difficult to interpret such knockin experiments because known homeostatic processes could have normalized transmission over the lifetime of the animal.

### **There must be a mechanism by which CaMKII/NMDAR complex can lead to the trans-synaptic structural growth that underlies late LTP**

LTP has an early phase that does not involve synapse growth and does not require protein synthesis; this is followed within  $\sim$ 1 hr by late LTP, which involves synapse growth and requires protein synthesis [39,40]. An important concept is the tag and capture model [41]. According to this model, strong stimulation leads to "tagging" of the stimulated synapse by addition of a

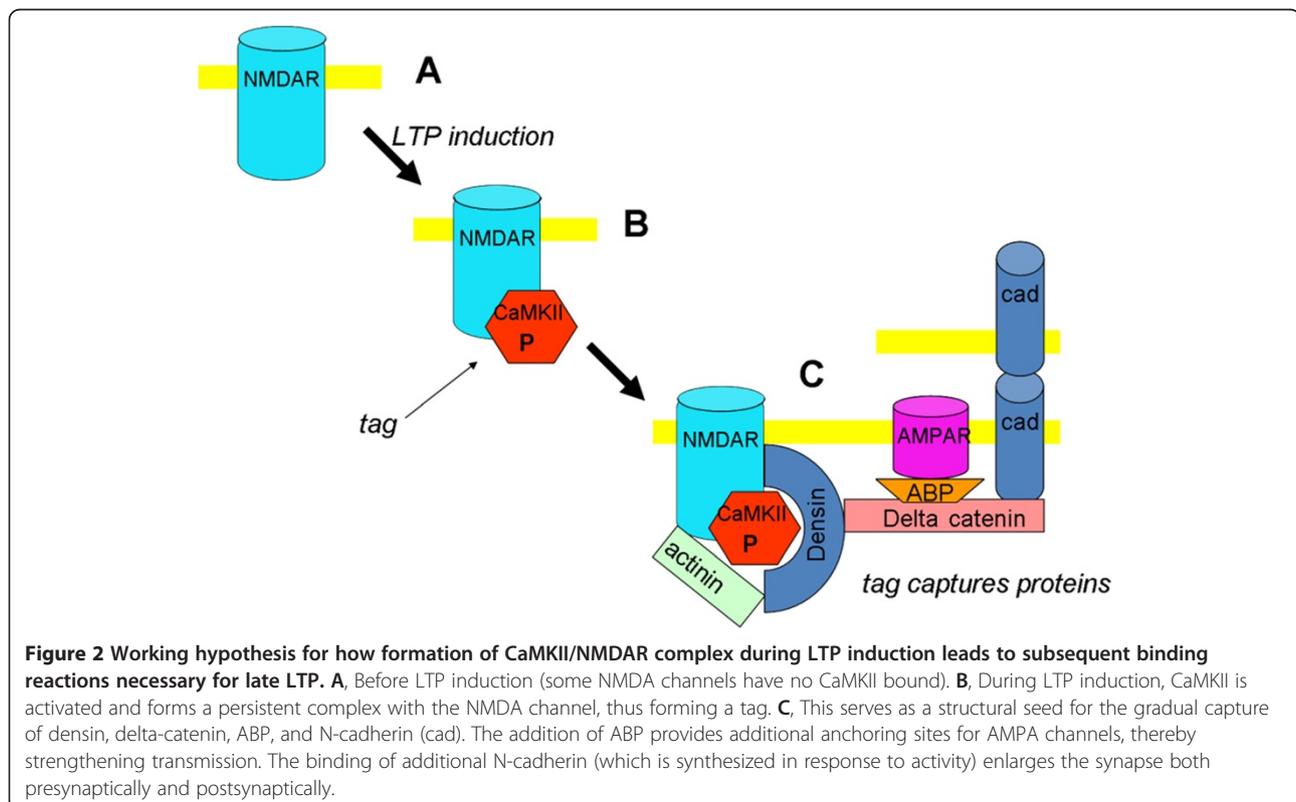
protein; this then serves to capture newly synthesized proteins, leading to late LTP at the activated synapse. Evidence suggests that CaMKII is the tag [42,43].

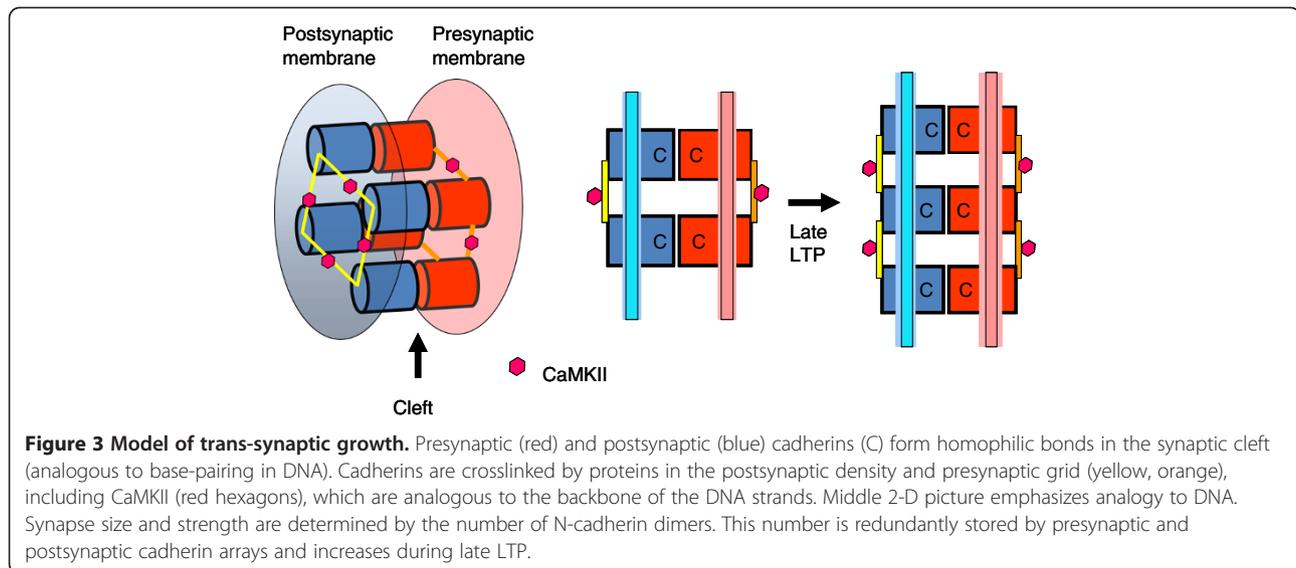
Relatively little is known about how proteins produce the synaptic growth that underlies late LTP. One area of progress has established a key role for N-cadherin, a homophilic adhesion molecule that forms a trans-synaptic linkage between the presynaptic and postsynaptic sides of the synapse. Pharmacological experiments, gene knockout experiments, conditional knockout experiments, and RNAi experiments all show that N-cadherin is required for late LTP (but not for early LTP) [44-46]. Furthermore, LTP induction causes synthesis of N-cadherin and its insertion into the synapse [44]. Finally, electron microscopy shows that overexpression of N-cadherin can increase synapse size [47]. Thus, although there are many forms of adhesion molecules at synapses, N-cadherin appears to be of central importance in late LTP.

There have been no previous suggestions about the cascade of biochemical events by which LTP induction results in the incorporation of N-cadherin into the synapse. Figure 2 shows a working hypothesis based on known binding interactions. It is proposed that the CaMKII/NMDAR complex acts as a structural seed for a series of binding reactions that gradually increase synapse size and strength, thereby accounting for late LTP. The following known binding interactions could be involved: densin-180 (and actinin) binds to the CaMKII

in the CaMKII/NMDAR complex [48,49]; delta-catenin binds to densin [50]; and N-cadherin binds to delta-catenin [51]. As noted above, overexpression of N-cadherin leads to synapse growth. We thus posit that the addition of N-cadherin to the synapse during late LTP similarly leads to synapse growth. An important constraint on any model of synapse growth is that it must be trans-synaptically coordinated, leading to precise registration of the edges of the presynaptic grid and postsynaptic density [52]. The role of the N-cadherin trans-synaptic dimers in organizing synaptic growth provides a simple explanation of this coordination (Figure 3).

Physiological analysis suggests that late LTP involves both addition of AMPA receptors and an increase in presynaptic release sites [53,54]. It is thus of interest that delta-catenin binds to AMPA-binding protein (ABP) [55] and may thereby increase the AMPA channel (notably GluA2) content of the synapse [56-58]. N-cadherin can also bind directly to GluA2 [59], see also [60]. There is relatively little that can be said about the presynaptic changes that lead to enhanced vesicle release. It is known that overexpression of postsynaptic N-cadherin affects presynaptic function [61], but how this happens is unclear. Perhaps presynaptic N-cadherin is brought into the synapse simply by binding to postsynaptic N-cadherin. Alternatively, insertion of presynaptic N-cadherin might depend on the presynaptic activation of CaMKII [62], translocation of CaMKII to the pre-





synaptic grid [63], and a protein linkage to N-cadherin [51] analogous to the postsynaptic linkage described in Figure 2.

It is noteworthy that Figure 3 suggests a limited analogy to DNA: just as in DNA, there are two crosslinked backbone structures attached to elements that link the two backbone structures by a pairing rule (in DNA, the rule is heterologous; here, it is homologous). The existence of two backbone structures provides redundant information storage. Thus the N-cadherin dimers could contribute to informational stability, just as base-pairing does in DNA. Specifically, if one member of an N-cadherin dimer was lost in the process of turnover, the existence of the other member could serve as a basis for repair. In this way, the number of N-cadherin dimers could be stable despite turnover of N-cadherin. This stability, in turn, could help to stabilize the CaMKII/NMDAR complex, as described in the next paragraph.

Although we have posited that the CaMKII/NMDAR complex brings about the capture of the protein complex that includes actinin, densin, delta-catenin, and N-cadherin, the complex, once established, may be important for the stabilization of CaMKII/NMDAR complex during protein turnover. During turnover of CaMKII, phosphorylated CaMKII holoenzymes may be removed from the NMDAR, leaving a pocket formed by densin and actinin. This pocket is relatively stable because it is held there by other parts of the complex that include the N-cadherin dimers (Figure 2). The phospho-CaMKII that left the pocket can be replaced by CaMKII that is either not phosphorylated (but with  $\text{Ca}^{2+}$ /calmodulin bound) or weakly phosphorylated. There, CaMKII can bind to GluN2B [11] and will become autophosphorylated as a result of stimulation by actinin [64] and GluN2B [16] and because PSD CaMKII is protected from phosphatase

[31] (probably because of the protein structure around bound CaMKII). This highly phosphorylated state then leads to tighter binding to the NMDAR [11]. In this way, the original tight complex of CaMKII bound to the NMDAR can be restored despite the molecular turnover.

This model provides an explanation for why overexpression of catalytically dead CaMKII can reverse memory (see next section). Specifically, this form (K42M) can bind to the NMDAR (albeit weakly), but not undergo the autophosphorylation necessary for tight binding to the NMDAR [11] and densin [65]. Weak binding to the NMDAR and densin may ultimately lead to dissociation of the entire complex, resulting in loss of LTP and memory.

Our model leads to testable predictions: that densin-180, delta-catenin, and N-cadherin are required for synapse growth and late LTP. As noted earlier, N-cadherin is required for late LTP (but not early LTP) and for synapse growth. Knockout of delta-catenin or densin does not block early LTP [58,66]; the effect on late LTP or synapse growth has not been tested.

### Effect of altering the CaMKII/NMDAR complex on behavioral tests of memory

Evidence that CaMKII signaling contributes strongly to the maintenance of nonspatial forms of memory has recently been published. One line of work comes from research on addiction in rats [67]. It was found that a persistent reversal of drug-induced sensitization could be produced by transient expression of a kinase-dead CaMKII mutant (K42M). In these experiments, animals were initially subjected to repetitive amphetamine exposure. Subsequently, transient virally mediated expression of K42M produced a persistent block of the normal long-lasting behavioral response to the drug (enhanced loco-

motion and drug self-administration). Because expression of K42M had ceased at the time of testing, the action of K42M must have been to reset a memory switch. In addition to the lack of catalytic activity and autophosphorylation, K42M displays an altered pattern of synaptic translocation in cultured neurons. While transient translocation is not affected [68,69], the strength of binding to the NMDAR is strongly decreased [21,70]. Thus, there is a reasonable basis for suspecting that K42M can erase a memory by acting as a dominant negative for stable CaMKII/NMDAR complex. A further indication that CaMKII is involved in the maintenance of behavioral memory comes from the experiments of [71]. They found that conditioned fear could be erased by transient post-learning inhibition of overexpressed CaMKII. However, the inhibition was animal-wide, so the location and physiological correlate of this effect are unclear.

Recent experiments have sought to test directly the role of the CaMKII/NMDAR complex in memory. To address this issue, [22] utilized a knockin mouse with mutations L1298A and R1300Q in GluN2B that interfere with that interferes with the formation of the CaMKII/NMDAR complex. A key finding was that spatial memory in the Morris water maze task, when measured at 1 or 3 days after the last training session, was greatly reduced (memory 1-2 hr after each training session was not reduced). Interpretation of the results is complicated by the fact that the mutation only produced a partial reduction in the basal CaMKII/NMDAR complex and only a ~50% reduction in LTP. It is thus possible that the learning that occurred in this task was due to this remaining ability of complex to form. It is also of interest to consider the possibility that CaMKII can form other complexes (i.e., not with NMDARs) that contribute to synaptic enhancement. Indeed, if presynaptic CaMKII is necessary for binding of N-cadherin and growth of the presynaptic active zone, the binding of CaMKII to a presynaptic protein, possibly Ca<sup>2+</sup> channels [72], could contribute to presynaptic structural changes during LTP. A further question is whether memory maintenance can be reversed by interfering with the CaMKII/NMDAR complex after learning. Only one study thus far deals with this issue [73]. It was found that a CN peptide injected into the cingulate cortex was able to reverse a form of central pain. Importantly, induction of central pain produced a large increase in the amount of CaMKII/NMDAR complex in PSDs of the cingulate, and this increase was reversed by CN.

### The PKM-zeta alternative

It is noteworthy that a major alternative hypothesis [74], that memory is stored by a persistent increase in PKM-

zeta, has substantial weaknesses, many of which have only recently become apparent. A critical test of the effect of PKM-zeta to reverse LTP maintenance has not been done. While it was shown that LTP in the slice could be reduced by ZIP, it was not shown that this reduction persisted after removal of ZIP [75]. In different experiments in which ZIP was transiently injected *in vivo*, the drug was still present 2 hr after injection, as shown by immunochemistry; no removal of drug was demonstrated [76]. Thus, the existing experiments do not distinguish between effects on LTP maintenance and effects on LTP expression. Furthermore, recent work casts strong doubt on whether ZIP has its effect by an action on PKM-zeta [1,77,78]. Importantly, recent work from the Sacktor laboratory demonstrates that the control peptide (scrambled ZIP) can also inhibit PKM-zeta and is only three times less effective than ZIP [79]. With such small differences in efficacy, it would take very precise administration of these peptides to produce a differential effect, but the concentrations used in *in vivo* experiments were highly imprecise (several orders of magnitude above the K<sub>d</sub>) [1]. Thus, the differential effects on ZIP and scrambled ZIP are probably due to an effect on some target other than PKM-zeta. Finally, knockout of this enzyme did not affect late LTP, memory, or the effect of ZIP [80] (but see [81]). Thus, the evidence that PKM-zeta is the molecular basis of memory is not compelling.

### Conclusions

The CaMKII/NMDAR complex is a promising candidate as the molecular basis of memory storage. Although there is increasing evidence that CaMKII and its binding partners form a molecular memory, additional work is required to prove this hypothesis. In particular, methods for studying the formation and persistence of the complex during actual LTP are needed. Furthermore, much additional work is required at the behavioral level to test the role of the CaMKII and the CaMKII/NMDAR complex in the persistence of memory. Most of the work on this complex has been done in the hippocampus. Thus, it will be of particular importance to determine whether interference with the complex in the hippocampus can reverse a hippocampal-dependent spatial memory.

An important unresolved question is whether the importance of CaMKII/NMDAR complex in LTP maintenance, as demonstrated in Figure 1, will be specific for CA1 or more generally applicable to synapses. One perspective on this question comes from analysis of the PSDs, which are generally isolated from whole brains. Structural analysis shows that these PSDs are nearly tight-packed with CaMKII [82]. Given that these PSDs are representative of the whole brain, it seems likely that a critical role for CaMKII will be a widespread property.

It has become increasingly clear that an important aspect of activity-dependent synaptic plasticity is the making and breaking of synaptic connections. Recent work shows that the ability of activity to stabilize synaptic connections is dependent on the CaMKII/NMDAR complex [83]. Thus, similar mechanisms may be involved in changes in synaptic strength and in stabilization of synaptic connections.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

JL and MS contributed equally to writing this review. Both authors read and approved the final manuscript.

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