Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2016 | 139 | 959–972

doi: 10.1111/jnc.13672

REVIEW

Interplay of enzymatic and structural functions of CaMKII in long-term potentiation

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Abstract

Since the discovery of long-term potentiation (LTP) about a half-century ago, Ca^{2+}/CaM-dependent protein kinase II (CaMKII) has been one of the most extensively studied components of the molecular machinery that regulate plasticity. This unique dodecameric kinase complex plays pivotal roles in LTP by phosphorylating substrates through elaborate regulatory mechanisms, and is known to be both necessary and sufficient for LTP. In addition to acting as a kinase, CaMKII has been postulated to have structural roles because of its extraordinary abundance and diverse interacting partners. It now is becoming clear that these two functions of CaMKII cooperate closely for the induction of both functional and structural synaptic plasticity of dendritic spines.

Keywords: CaMKII, cytoskeleton, synaptic plasticity.

J. Neurochem. (2016) **139**, 959–972.

This article is part of a mini review series: "Synaptic Function and Dysfunction in Brain Diseases".

CaMKII is a serine (S)/threonine (T)-specific protein kinase activated by the Ca^{2+}/calmodulin (CaM) complex, consisting of catalytic, regulatory (autoinhibitory/CaM binding) and association domains. Under basal conditions, CaMKII is subject to autoinhibition via an interaction between its kinase and regulatory domains in the absence of Ca^{2+}/CaM. In this 'closed' conformation, a pseudosubstrate segment and T286 in the regulatory domain bind to the S (substrate binding) site and T (T286 binding) site in the catalytic domain, respectively, thus preventing the access of substrates. A recent structural study demonstrated that the compact arrangement of inactive CaMKII holoenzyme renders the CaM-binding domain inaccessible, and the strength of the autoinhibitory interaction (degree of 'compactness') depends on the length of linker between the kinase and association domain (Chao *et al.* 2011).

The elevation of Ca^{2+} levels during the induction of synaptic plasticity activates CaMKII. Binding of Ca^{2+}/CaM to the regulatory domain disrupts autoinhibition and places CaMKII in an 'open' conformation, thereby making T286 available for phosphorylation. Importantly, because of its dodecameric structure, the CaMKII holoenzyme can have multiple activation states depending on the intensity and duration of the Ca^{2+} stimulus (Lisman *et al.* 2002). With a weak and short Ca^{2+} stimulus, bound Ca^{2+}/CaM detaches from CaMKII immediately after Ca^{2+} levels subside, and CaMKII becomes inactive before T286 is phosphorylated.

Received March 15, 2016; revised manuscript received May 9, 2016; accepted May 10, 2016.

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Abbreviations used: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; FRET, Förster resonance energy transfer; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GluA1/2, AMPA-type glutamate receptor subunits 1 and 2; GluN1, 2A and 2B, NMDA-type glutamate receptor subunits 1, 2A and 2B; GTPase, guanosine triphosphatase; PDZ, Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and Zonula occludens-1 protein (Zo-1); KIF17, kinesin-like protein 17; LTD, long-term depression; LTP, long-term potentiation; NMDA, N-methyl-D-aspartate; SAP97, synapse-associated protein 97; SynGAP, synaptic GTPase-activating protein.

With a stronger and longer Ca^{2+} stimulus, two adjacent CaMKII monomers are simultaneously occupied by Ca^{2+}/CaM. Under this condition, one subunit acts as a substrate for the other, resulting in T286 phosphorylation. Once one subunit is phosphorylated, the subsequent phosphorylation within the holoenzyme is more likely to occur than the initial phosphorylation, as the Ca^{2+} level required for the second phosphorylation is lower than that required for the initial phosphorylation. So, CaMKII remains active even when Ca^{2+} levels return to basal levels until it is dephosphorylated. If the number of phosphorylated subunits is above a threshold and the rate of phosphorylation exceeds that of dephosphorylation, then CaMKII activity is sustained. This bistability of the CaMKII molecule has been predicted from modeling studies and a recent experimental study using purified CaMKII and phosphatase confirmed this view (Michalski 2013; Urakubo *et al.* 2014).

There are excellent reviews which contain the most up to date information regarding the regulation and roles of CaMKII during synaptic plasticity (Coultrap and Bayer 2012; Lisman *et al.* 2012; Hell 2014; Shonesy *et al.* 2014; Herring and Nicoll 2016). In this review, we will specifically focus on how enzymatic and structural functions of CaMKII cooperate with each other to facilitate synaptic plasticity of dendritic spines.

Early views of the roles of CaMKII in LTP

Because of the above unique regulatory mechanisms, CaMKII has been hypothesized to act as 'a frequency detector' which integrates multiple Ca^{2+} pulses, and 'a memory molecule' which remembers the past history of synaptic activation (De Koninck and Schulman 1998). Multiple proteins have been proposed as substrates of CaMKII crucial for the expression of long-term potentiation (LTP). Different views have emerged during the last few decades, some require revision while others were supported by later research. In the subsequent sections, we will highlight the different views of CaMKII-mediated regulation of postsynaptic proteins.

Significance of AMPA-type glutamate receptor phosphorylation

LTP is characterized by an increase in both number and function of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor (AMPA receptor). Therefore, it is natural to hypothesize that the AMPA receptor is a target of CaMKII itself. In the CA1 region of the hippocampus where LTP has been most extensively studied, AMPA-type glutamate receptor subunits 1 and 2 (GluA1/2) heteromeric AMPA receptor accounts for ~ 80% of synaptic, and 95% of extrasynaptic, AMPA receptor (Lu *et al.* 2009). GluA1 has multiple phosphorylation sites, and the phosphorylation of S831 on the intracellular carboxyl tail is known to be increased during LTP (Barria *et al.* 1997; Lee *et al.* 2000). CaMKII enhances AMPA receptor function by phosphorylating S831 of GluA1, resulting in an increase in receptor conductance (Derkach *et al.* 1999). Accordingly, GluA1 knock-in mice harboring a S831 to aspartate (D) mutation showed an increased channel conductance (Kristensen *et al.* 2011). However, preventing the phosphorylation of S831 with an alanine (A) mutation alone did not affect LTP and an additional S845A mutation was required to see an impairment in LTP (Lee *et al.* 2010), suggesting a potential interaction between these two sites.

Hosokawa *et al.* (2015) provided evidence that cast doubt on this model. Using Phos-tag sodium dodecyl sulfate–polyacrylamide gel electrophoresis, a unique method that allows detection of the stoichiometry of site-specific phosphorylation, they could detect only an extremely low proportion of GluA1 phosphorylated at S831 and S845 (< 1%) in both control and stimulated hippocampi experiencing chemical LTP or learning, a measure which is much lower than predicted from previous models. Additionally, the authors discovered that phosphorylation was much higher in postnatal day 1 neurons than in mature adult tissue. This suggests that phosphorylation of AMPA receptors may play a role during development. However, considering that multiple studies suggest the role of GluA1 phosphorylation in synaptic plasticity and learning/memory including the study of knock-in of S831A and S845A mutation (Lee *et al.* 2003), more elaborate investigation in terms of the time course of AMPA receptor phosphorylation in animals of different age will be required.

In addition to directly phosphorylating AMPA receptor, CaMKII increases the number of synaptic AMPA receptors by phosphorylating the carboxyl tail of stargazin, an AMPA receptor auxiliary subunit protein. Under unphosphorylated conditions, the carboxyl tail of stargazin is tethered to the inner leaflet of the plasma membrane via electrostatic interaction from the positively charged arginine (R) residues. Phosphorylation of stargazin by CaMKII at the overlapping serine and threonine neutralizes the charge and interferes with this interaction, allowing PSD-95 to bind to the PDZ-binding motif of stargazin, which in turn immobilizes the AMPA receptor at synaptic sites (Opazo *et al.* 2010; Sumioka *et al.* 2010). This phosphorylation leads to the synaptic insertion of AMPA receptor from extrasynaptic sites.

Duration of CaMKII activation in LTP

In the traditional view, once CaMKII is activated, it remains in an active conformation throughout the LTP maintenance phase, which forms the basis of the hypothesis that CaMKII is a memory molecule. Indeed, Barria *et al.* (1997), Fukunaga *et al.* (1993) and Ouyang *et al.* (1997)

demonstrated long-lasting CaMKII activity and autophosphorylation after LTP induction. However, recent results using Camui, a Förster resonance energy transfer (FRET)-based CaMKII sensor, challenged the view that persistent CaMKII activation continues after LTP induction (Takao *et al.* 2005; Lee *et al.* 2009). Contrary to previous reports showing a long-lasting CaMKII autophosphorylation after LTP induction, the authors showed that CaMKII activity only lasts ~ 1 min within the stimulated spine during LTP induction by two-photon laser-mediated photolysis of caged glutamate. Optical monitoring of CaMKII activity has the advantage of greater spatiotemporal resolution over more traditional histological approaches, for example, immunoblot of tissue homogenate, but it still has technical limitations in detecting small amounts of activated CaMKII within dendritic spines. For example, when CaMKII binds to the carboxyl tail of GluN2B, it becomes locked in an active conformation, which is believed to be important for LTP (Bayer *et al.* 2001; Barria and Malinow 2005) (see below for details). Such fractions may not be detected by Camui as the amount is estimated at 0.2% of the total amount of CaMKII in the spine (Feng *et al.* 2011). Also, as Hell (2014) indicated, Camui may not represent actual catalytic activity of CaMKII, because the Camui signal is more strongly influenced by T286 autophosphorylation than Ca^{2+}/CaM (Lee *et al.* 2009), while CaMKII shows maximal activity with Ca^{2+}/CaM and without T286 autophosphorylation (Braun and Schulman 1995; Coultrap *et al.* 2010). However, a recent report demonstrated that the fast decay in Camui signal is not because of the dephosphorylation of T286, but rather it represents a detachment of Ca^{2+}/CaM complex (Otmakhov *et al.* 2015). Therefore, the relationship between activity, conformation and T286 phosphorylation of CaMKII needs further investigation, and care should be taken when interpreting results obtained with Camui.

Structural roles of CaMKII in LTP

The importance of understanding the role of CaMKII, however, remains unchanged given its requirement in LTP (Silva *et al.* 1992; Pettit *et al.* 1994). There has been one long-standing mystery of CaMKII, its high abundance at the synapse. Estimates suggest that CaMKII accounts for 1–2% of total brain protein (Lisman *et al.* 2002), and 2–6% to even 10–30%, depending on the report, in the PSD (Kennedy *et al.* 1983; Peng *et al.* 2004; Chen *et al.* 2005), thus overwhelming typical scaffolding proteins such as PSD-95 (Peng *et al.* 2004; Dosemeci *et al.* 2007; Sheng and Hoogenraad 2007) and rivaled only by a few canonical cytoskeleton proteins such as actin and tubulin (Peng *et al.* 2004). Therefore, there has been long-standing speculation that CaMKII has structural functions by itself, in addition to the enzymatic activity.

Indeed, LTP induction simultaneously drives rapid and persistent enlargement of dendritic spines along with enhanced AMPA receptor transmission, which is termed structural LTP (sLTP) (Matsuzaki *et al.* 2004; Okamoto *et al.* 2004). At the same time, CaMKII and other proteins translocate into the enlarged spine structure (Bosch *et al.* 2014). Inhibition of CaMKII does not abolish the early phase but blocks the persistent phase of sLTP. Therefore, how structural and enzymatic functions of CaMKII are involved in both electrophysiologically measured functional LTP and structural LTP needs to be fully elucidated. This raises a question on a new level: Are these two functions of CaMKII, structural and enzymatic, independent of each other or do they interact in some way? Indeed, outcomes that follow CaMKII activation are not independent, but closely linked with each other to change the efficiency of synaptic transmission and the structure of a synapse.

Characteristics of CaMKII as a structural component

Compared to other enzymes or adaptor/scaffold proteins, CaMKII has several characteristics to undertake this unique role in inducing synaptic plasticity. First, the dodecameric structure of CaMKII allows it to interact with multiple proteins simultaneously and work as a scaffold protein (Walikonis *et al.* 2001; Robison *et al.* 2005). Thus, CaMKII not only can provide physical links between proteins, but can also act as a hub where multiple signaling pathways crosstalk. Second, each monomer in the CaMKII holoenzyme is an enzyme that phosphorylates its own substrates and initiates downstream signaling. Unlike other 'enzyme-only' and 'scaffold-only' proteins, this unique 'all-in-one' property of CaMKII enables faster and more efficient local signaling. CaMKII not only physically anchors proteins, but acts upon them as well. Third, a recent structural study revealed that two neighboring kinase domains are organized in dimers in the holoenzyme, leading to the notion that subunits in the same dimer pair are likely to have the same activity states (Rosenberg *et al.* 2005). At the same time, each dimer pair can be separately regulated so that each holoenzyme can have multiple activation states. This enables the CaMKII holoenzyme as well as each dimer pair in a single holoenzyme to carry out different functions depending on different conditions, hence greatly widening its role in a diverse range of environments. Finally, on top of individual regulation of each subunit, the subunit composition of each holoenzyme is heterogenous. Although CaMKIIα homomeric complexes can be found, the majority of CaMKII in the brain exists as heteromeric complexes comprising CaMKIIα and β, the two predominant isoforms in the brain (Bennett *et al.* 1983; Kanaseki *et al.* 1991; Brocke *et al.* 1999). Importantly, although these two isoforms show high amino acid homology and similar domain structures, they have their own characteristics. The Ca^{2+}/CaM affinity of CaMKIIβ is ~ 10 times higher than that of CaMKIIα

(Brocke *et al.* 1999), so CaMKIIβ expression increases in response to a reduction of neuronal activity, whereas CaMKIIα dominates during elevated levels of neuronal activity (Thiagarajan *et al.* 2002). Also, some proteins or part of a protein show isoform-specific binding to CaMKII, for example, actin and Arc bind specifically to CaMKIIβ (Fink *et al.* 2003; Okamoto *et al.* 2007; Lin and Redmond 2008; Okuno *et al.* 2012). The C-terminus of densin180 interacts only with CaMKIIα, while its central domain binds to both α and β isoforms (Strack *et al.* 2000; Walikonis *et al.* 2001; Jiao *et al.* 2011). Therefore, CaMKII has another method to diversify its interaction profile by changing the ratio between these isoforms depending on different conditions. Interestingly, the α:β ratio in the adult forebrain is ~ 3 : 1, while this ratio is reversed in the cerebellum (Erondu and Kennedy 1985). Additionally, the ratio changes during development along with a change in domain composition because of alternative splicing (Brocke *et al.* 1995). Indeed, it was shown that the ratio between CaMKIIα and β is important for the modulation of rebound potentiation, a form of plasticity exhibited at inhibitory synapses of cerebellar Purkinje cells (Nagasaki *et al.* 2014).

These unique features of CaMKII make it an ideal candidate protein to underlie synaptic plasticity. We will describe how this is accomplished through the cooperation of enzymatic and structural functions of CaMKII in the following sections (Fig. 1).

Actin

Actin is a major cytoskeletal protein in the spine serving not only as a structural framework but also as a scaffold that recruits proteins to link signaling between the synapse surface and cytoplasm (Okamoto *et al.* 2009). The equilibrium between monomeric G-actin and polymeric F-actin within the spine shifts during LTP and long-term depression (LTD) (Okamoto *et al.* 2004), implying a potential role as a synapse tag during synaptic plasticity (Okamoto *et al.* 2009).

The binding of CaMKII to actin is attributed to the actin-binding domain of CaMKIIβ located between the regulatory and association domain. With this, the CaMKII holoenzyme containing CaMKIIβ binds to and crosslinks F-actin *in vitro* and in neurons (Shen and Meyer 1999; Fink *et al.* 2003; Okamoto *et al.* 2007; Lin and Redmond 2008). CaMKIIβ KO mice show impaired hippocampal LTP and hippocampus-dependent learning (Borgesius *et al.* 2011). However, in the same study, LTP and learning were normal in a CaMKIIβ alanine 303 to arginine mutant knock-in mouse, in which the CaM binding is impaired thus catalytically inactive, while F-actin binding and bundling activity is intact. This suggests that the phenotype of the CaMKIIβ KO mouse is not because of the absence of catalytic activity but because of impaired spine targeting of CaMKIIα as shown in dissociated hippocampal neurons from KO mice. CaMKIIβ also shows isoform-specific morphological effects on dendritic arborization, neurite motility and synapse formation that CaMKIIα does not have, and these effects are dependent on actin binding to CaMKIIβ (Fink *et al.* 2003). Interestingly, the amount of CaMKIIβ available to bind to F-actin gradually increases during development due to alternative splicing of the actin-binding domain (Brocke *et al.* 1995), suggesting that the CaMKII-actin interaction is dynamically regulated and plays an important role in synaptic plasticity and neuronal development.

The CaMKII-actin interaction not only contributes to the localization of CaMKII within spines, but also maintains the structure of mature spines with a clear head and neck, by stabilizing the actin cytoskeleton (Okamoto *et al.* 2007; Lin and Redmond 2008; Kim *et al.* 2015). Upon down-regulation of CaMKIIβ, but not CaMKIIα, the proportion of mature spines is reduced. The phenotype is rescued by a kinase activity-deficient CaMKIIβ lysine 43 to arginine mutant, indicating that the structural properties of CaMIIβ rather than the catalytic activity is responsible for stabilization of mature spines (Okamoto *et al.* 2007). This CaMKII-mediated actin stabilization effect is attributed to the inhibition of interaction with actin regulating proteins most likely by steric interference. We showed that binding to unphosphorylated CaMKII inhibits access of actin-regulating proteins such as gelsolin, cofilin and the Arp2/3 complex to actin, thereby inhibiting their activity (Fig. 1, Circle 1) (Kim *et al.* 2015).

The interaction of CaMKII with F-actin is negatively regulated by neuronal activity (Shen and Meyer 1999; Lin and Redmond 2008; Kim *et al.* 2015). This mechanism appears to be important for inducing synaptic plasticity both functionally and structurally. During LTP induction, activated CaMKII is released from F-actin, which is mediated by autophosphorylation of the actin-binding domain of CaMKIIβ (Fig. 1, Circle 2). Activation of purified CaMKIIβ *in vitro* with Ca^{2+}/CaM results in extensive phosphorylation of serines and threonines in the actin-binding domain, and dissociated hippocampal neurons show similar levels of phosphorylation following chemical LTP (Kim *et al.* 2015). Interestingly, among the four subregions of the actin-binding domain derived from alternative splicing, the first subregion whose insertion is regulated during development (Brocke *et al.* 1995) is the most important for actin binding, emphasizing again the importance of this region in CaMKIIβ function.

Of note, CaMKII detachment from F-actin is indispensable for the induction of LTP. When this detachment was blocked by alanine mutations of all phosphorylatable serines and threonines in the actin-binding domain, both functional and structural LTP were impaired without impairing kinase activity *per se*. But although necessary, the detachment itself is not sufficient for LTP induction, such that signaling pathway(s) downstream of glutamate receptors must accompany the CaMKII detachment from F-actin for proper LTP

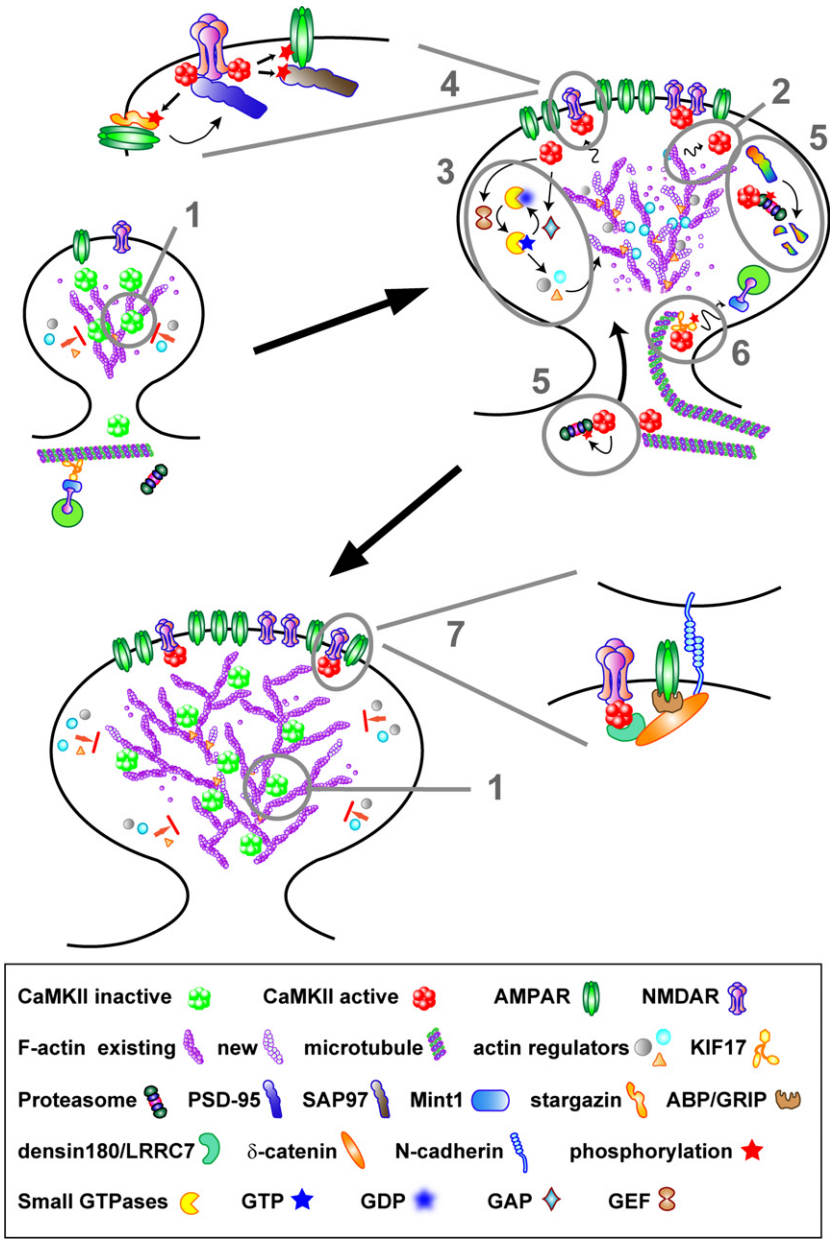

Fig. 1 Contribution of CaMKII to long-term potentiation (LTP) by cooperation of enzymatic and structural functions. In its basal state, inactive CaMKII binds the actin cytoskeleton through an actin-binding domain on the β subunit and stabilizes it by limiting access of actin regulators (orange arrow and red line in small spine on the left). Activated CaMKII during LTP induction by Ca^{2+} influx facilitates the remodeling of the postsynaptic structure in several ways by the cooperation of its enzymatic and structural functions. (1) Inactive CaMKII binds to the actin cytoskeleton and protects it from actin regulators. (2) Autophosphorylation of the actin-binding domain of the β isoform detaches CaMKII from F-actin, leading to the reorganization of the actin cytoskeleton by allowing access of actin regulators to actin. (3) Active CaMKII regulates the activity of Rho family small GTPases by phosphorylating GTPase-activating protein (GAP) and guanine nucleotide exchange factor (GEF) proteins, which results in modification of actin through downstream effectors. (4) Active CaMKII bound to NMDA receptors increases both number and function of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor by phosphorylating AMPA-type glutamate receptor subunits 1 (GluA1), SAP97 and stargazin. (5) Active CaMKII recruits proteasomes to stimulated spines by direct binding and enhances their activity by phosphorylation, which facilitates degradation of proteins unnecessary for LTP. (6) CaMKII contributes to LTP by activity-dependent interaction with microtubules. Also, active CaMKII phosphorylates KIF17, releasing necessary cargo protein for LTP such as GluN2B into stimulated spines. (7) The CaMKII–NMDA receptor complex provides more slots for AMPA receptors and strengthens transsynaptic connections through a series of interactions with densin180/LRRC7, δ-catenin, AMPA-binding protein/GRIP and N-cadherin.

induction. This can be regarded as a 'double verification' mechanism to avoid unnecessary changes in synaptic transmission and spine structure. This also contributes to the synapse specificity of LTP.

In addition to regulating actin through direct binding, CaMKII regulates actin by phosphorylating regulators of the Rho family of small GTPases, resulting in reorganization of actin within the spine (Saneyoshi and Hayashi 2012) (Fig. 1, Circle 3). Signaling pathways triggered by CaMKII activation during LTP are known to regulate the activity of Rac1, Cdc42 and RhoA, the three most abundant members of the Rho family, and ultimately induces changes in actin organization in a spine through effector molecules such as cofilin and profilin (Saneyoshi and Hayashi 2012). These three GTPases have distinct roles in neurons: while Rac1 and Cdc42 are positive regulators of spine formation and maintenance, RhoA exerts the opposite effect (Nakayama *et al.* 2000; Tashiro *et al.* 2000; Scott *et al.* 2003; Ahnert-Hilger *et al.* 2004). The activity of Rho GTPase family proteins are regulated by three classes of proteins: guanine nucleotide exchange factor (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors. Among them, CaMKII directly or indirectly affects the activity of GEFs and GAPs by phosphorylation, and thus modulates the activity of Rho family small GTPases.

CaMKII activates Rac1 by phosphorylating Kalirin-7 and T-cell lymphoma invasion and metastasis 1, both of which are Rac-GEFs (Fleming *et al.* 1999; Buchanan *et al.* 2000; Xie *et al.* 2007). Although *Kalirin-7*-specific knockout and *Kalirin* total null mice show a modest reduction in hippocampal LTP (Ma *et al.* 2008; Xie *et al.* 2011), overexpression of Kalirin-7 increases AMPA receptor current via a process that is dependent on synaptic activity and CaMKII. Moreover, CaMKII-mediated synaptic potentiation is abolished by expression of a non-phosphorylatable Kalirin-7 containing a T95A mutation, which is the CaMKII phosphorylation site (Xie *et al.* 2007; Herring and Nicoll 2016), suggesting that Kalirin-7 acts as a downstream substrate for CaMKII during LTP. On the other hand, CaMKII recruits RhoA-specific GEF Lfc into spines by phosphorylating spinophilin (Grossman *et al.* 2004; Ryan *et al.* 2005). All these signaling pathways initiated by CaMKII-mediated phosphorylation converge to modify the actin cytoskeleton in a spine by triggering actin regulators (such as cofilin and profilin) through their upstream regulator proteins such as p21-activated kinase, LIM domain kinase and Rho-associated protein kinase.

FRET-based sensors have made it possible to monitor the detailed spatiotemporal activity profiles of Rho family small GTPases in a single spine undergoing structural plasticity (Murakoshi *et al.* 2011). The authors showed that both RhoA and Cdc42 are rapidly activated during structural LTP in a spine stimulated by two-photon glutamate uncaging pulses. This initial peak in activity decays in about 5 min followed by a persistent activation (~ 30 min), similar to the spine volume change during sLTP. Interestingly, these two small GTPases showed different behaviors based on activity patterns. Activated RhoA diffused out of the stimulated spine and spread along the dendrite, whereas Cdc42 remained confined to the stimulated spine. In subsequent experiments using small hairpin ribonucleic acid and inhibitors of GTPase signaling, the authors demonstrated that the RhoA-Rho-associated protein kinase pathway is required for both the initial (transient) and sustained phases of the spine enlargement, while the Cdc42-p21-activated kinase pathway is required only for the sustained phase. This observation highlights the importance of these two pathways in sLTP. Importantly, both RhoA and Cdc42 activation was blocked by a CaMKII inhibitor (Murakoshi *et al.* 2011).

Thus, CaMKII connects transient Ca^{2+} signaling to long-term changes in spine structure during synaptic plasticity by two mechanisms, one by modulating the activity of Rho family small GTPases, another by modulating its F-actin binding and stabilizing capability in an activity-dependent manner.

NMDA receptors

CaMKII translocates from the dendritic shaft to spines and the PSD in an activity-dependent manner (Shen and Meyer 1999; Dosemeci *et al.* 2001; Feng *et al.* 2011; Bosch *et al.* 2014). This spine enrichment and translocation are mediated by interaction with proteins in spines, and NMDA receptor subunits are one of the most important recruiters of CaMKII (Fig. 1, Circle 4). CaMKII binds to GluN1, 2A and 2B, but the interaction with GluN2A is much weaker than that of the other two subunits (Strack and Colbran 1998; Leonard *et al.* 1999). CaMKII binds to the region between residue 845 and 861 of GluN1 (the C0 domain), at the C-terminal cytoplasmic region close to the membrane. This binding domain was initially known to be involved in CaM-mediated negative feedback of NMDA receptor function to prevent excess Ca^{2+} influx (Ehlers *et al.* 1996), and is also shared by α-actinin. Under control conditions, Ca^{2+}-free CaM and α-actinin are bound to the C0 domain of GluN1 and CaMKII competes with α-actinin for binding to GluN1. Upon NMDA receptor activation, conformational change in Ca^{2+}-loaded CaM displaces α-actinin, resulting in simultaneous binding of CaMKII and Ca^{2+}-CaM to the C0 domain (Merrill *et al.* 2007), thereby providing a mechanism of Ca^{2+}-dependent NMDA receptor inactivation (Zhang *et al.* 1998; Krupp *et al.* 1999). It is unknown exactly how CaMKII desensitizes the receptor, whether it is purely through binding or if any phosphorylation reaction is involved.

GluN2B binding of CaMKII is also activity-dependent, through either autophosphorylation at T286 or Ca^{2+}/CaM binding which induces a high-affinity interaction of CaMKII with GluN2B (Strack and Colbran 1998; Bayer *et al.* 2001; Leonard *et al.* 2002). T286-phosphorylated CaMKII binds to

the GluN2B cytoplasmic domain more than 10 times tighter than to GluN1 and GluN2A (Strack and Colbran 1998). CaMKII binds to two sites on GluN2B: one around amino acid residue 1290–1309 which does not require T286 phosphorylation, and another around amino acid residue 839–1120 which does require T286 phosphorylation. Importantly, interaction with the GluN2B region around 1290–1309 'locks' the enzyme in an active state, rendering it persistently active regardless of the phosphorylation states even after the dissociation of Ca^{2+}/CaM (Bayer *et al.* 2001). This is because GluN2B binds to the 'T site' in the catalytic domain of CaMKII that is normally occupied by the regulatory domain, and unphosphorylated T286 renders the kinase inactive through autoinhibition. Thus, GluN2B binding has a similar effect to T286 phosphorylation, maintaining the open conformation of CaMKII. Interaction with GluN2B also increases CaMKII's affinity for CaM and induces CaM trapping, allowing CaMKII to stay active longer in the synapse by inhibiting the phosphorylation of T305, which induces dissociation of CaMKII from the synapse (Shen *et al.* 2000; Elgersma *et al.* 2002; Weeber *et al.* 2003). This CaMKII-GluN2B interaction plays essential roles in LTP and learning, such that inhibition of the formation of this complex leads to impairments in LTP and learning (Barria and Malinow 2005; Zhou *et al.* 2007; Foster *et al.* 2010; Halt *et al.* 2012).

Once formed, the CaMKII-NMDA receptor complex is stable and persistent (Bayer *et al.* 2006). Indeed, PSD-associated CaMKII remains even 60 min after LTP induction (Otmakhov *et al.* 2004). It is believed that NMDA receptor-bound, persistently active CaMKII which translocates into the PSD during LTP induction is important for LTP maintenance and information storage. Using a knock-in animal of GluN2B L1298A/R1300Q that prevents interaction of GluN2B with the T-site of CaMKII, Halt *et al.* (2012) demonstrated that the S831 phosphorylation of AMPA receptor GluA1 subunit is reduced, indicating that specific anchoring of CaMKII at the vicinity of Ca^{2+} source is required for maintaining normal phosphorylation level of GluA1. Hamilton *et al.* (2012) found that the activity-induced spine growth is also impaired in this animal. These results both support the importance of interaction between CaMKII and GluN2B in exhibiting normal synaptic plasticity. The requirement of a dual activation mechanism (T286 phosphorylation and GluN2B binding) for CaMKII can be an initial test to identify synapses which will ultimately be potentiated. As structural and functional changes to a synapse are time- and energy-consuming processes, these verification mechanisms can prevent the unnecessary waste of cellular resources.

A recent review by Lisman and Raghavachari (2015) details the potential underlying molecular mechanism of this stable LTP maintenance. The key feature which maintains the 'on' state of CaMKII is the ability to have intersubunit phosphorylation of a holoenzyme. By this mechanism, a CaMKII holoenzyme can restore the loss of T286 phosphorylation by phosphatase activity or subunit exchange by protein turnover. Modeling studies showed that the phosphorylation status of CaMKII is maintained by intersubunit autophosphorylation in the PSD (Lisman 1985; Miller *et al.* 2005). Importantly, in *in vitro* reconstituted system using purified proteins, Urakubo *et al.* (2014) showed that this persistent CaMKII activity (measured by T286 phosphorylation) was achieved only when GluN2B-derived peptide is present. Moreover, GluN2B-bound CaMKII has an increased affinity to ATP and is protected from dephosphorylation of T286 from protein phosphatase-1 (Pradeep *et al.* 2009; Cheriyan *et al.* 2011).

The CaMKII-GluN2B interaction results in multiple events each of which cooperatively contributes to LTP. First, by binding to NMDA receptors, CaMKII is recruited to the PSD where it can phosphorylate other synaptic substrates more easily. GluN2B-bound CaMKII may have better access to its substrate proteins such as GluA1, stargazin and SAP97 than free cytosolic CaMKII, leading, for example, to an increase in channel conductance (GluA1), binding to PSD-95 and thus immobilization at synaptic sites (stargazin) and synaptic accumulation of SAP97 (and AMPA receptor). Therefore, the physical interaction with GluN2B followed by the catalytic action of CaMKII upon LTP induction results in an increase in synaptic transmission.

Second, the CaMKII–GluN2B complex acts as a structural seed for generating bigger protein complexes that accommodate more AMPA receptors and enhance the trans-synaptic connection. Recently, Sanhueza and Lisman (2013) proposed that the CaMKII–NMDA receptor complex can be a 'molecular tag' in the synapse tag and capture hypothesis that Frey and Morris (1997) suggested. The CaMKII–NMDA receptor complex formed by LTP induction (or learning) at stimulated spines triggers a series of protein capturing reactions consisting of Densin180/α-actinin/δ-catenin/AMPA-binding protein/AMPA receptor. This eventually results in an increase in synapse size and strength mediated by trans-synaptic linkage between pre- and postsynaptic N-cadherin, thereby accounting for late LTP (Fig. 1, Circle 7) (Sanhueza and Lisman 2013). Although experimental evidence is still missing, this hypothesis emphasizes the importance of the structural role of CaMKII in LTP maintenance.

Also, NMDA receptor binding enables CaMKII to react more sensitively to local Ca^{2+} level changes at the opening of the Ca^{2+} channel. This, in combination with the ring-shaped structure of the CaMKII holoenzyme, allows efficient recovery of the activity of GluN2B-bound CaMKII. Even when a few subunits are dephosphorylated at T286 sites, they can be quickly rephosphorylated by neighboring subunits even at suboptimal Ca^{2+} concentrations, because of the ring structure and proximity to Ca^{2+} entry and the resulting elevated Ca^{2+} nanodomains.

Another line of evidence supporting the structural role of the CaMKII–GluN2B complex in LTP comes from an inhibitor experiment. TatCN21 peptide, derived from CaMKII inhibitory protein CaMKIIN, can inhibit both enzymatic activity and the GluN2B interaction by binding to the T site of CaMKII. Interestingly, the concentration needed to affect the two mechanisms is different. In hippocampal slices, 5 μM is sufficient for inhibition of enzymatic activity (Buard *et al.* 2010), whereas 20 μM is required for disruption of the CaMKII–GluN2B interaction (Sanhueza *et al.* 2011). Taking advantage of this difference, it was reported that low concentrations of tatCN21 which is sufficient to inhibit catalytic activity, inhibited LTP only when administered before LTP induction but not after, consistent with a study using a CaMKII FRET sensor, Camui (Lee *et al.* 2009). This indicates that the kinase activity is not required at later time points following LTP induction. However, a high concentration of tatCN21 can still reverse LTP (Sanhueza *et al.* 2011). This indicates that it is the CaMKII–GluN2B complex formation, rather than CaMKII's catalytic activity, that is necessary for the maintenance of LTP.

Proteasomes

Considering that LTP and LTD are changes in both the efficiency of synaptic transmission and physical size of a synapse, it is not surprising that proteasome activity is required to remove unnecessary proteins and help reorganize synaptic composition and structure in both cases (Colledge *et al.* 2003; Fonseca *et al.* 2006). Accordingly, consolidation and retrieval of memory also rely on proteasome activity (Lopez-Salon *et al.* 2001; Lee *et al.* 2008). Proteasomes show rapid translocation to spines upon synaptic activation (Bingol and Schuman 2006), and it was demonstrated that CaMKIIα recruits proteasomes to activated spines (Bingol *et al.* 2010) (Fig. 1, Circle 5). For proteasome interaction and redistribution, CaMKIIα must undergo T286 autophosphorylation. Importantly, degradation of endogenous proteasome substrates within the spine was impaired when CaMKIIα translocation was inhibited by a CaM-binding-deficient threonine 305 and 306 to aspartate mutant or a T-site blocking isoleucine 205 to lysine mutation, and again, kinase activity was not required for degradation. Thus, CaMKIIα acts as a scaffolding protein to specifically target proteasomes to activated spines, and promotes local protein degradation to support synaptic plasticity.

Upon binding, CaMKIIα phosphorylates S120 of proteasome subunit Rpt6, enhancing its activity (Djakovic *et al.* 2009). Phosphorylation of the same site was shown to be important for activity-induced outgrowth of new spines (Hamilton *et al.* 2012). Similarly to LTP, spine outgrowth also requires activation of NMDA receptors, CaMKII and the interaction between CaMKII and GluN2B. Also, a recent finding shows that CaMKII-dependent proteasome activation in the amygdala is important for memory reconsolidation (Jarome *et al.* 2016). Therefore, it is possible that CaMKII structurally recruits proteasomes to activated spines in parallel with enhancing its activity for LTP as well as new spine formation, and affects learning and memory.

Arc

Arc/Arg3.1 is one of the immediate-early-gene family members, and promotes AMPA receptor endocytosis through interaction with endophilin and dynamin (Chowdhury *et al.* 2006). Accordingly, Arc is involved in several forms of LTD (Plath *et al.* 2006; Park *et al.* 2008; Waung *et al.* 2008; Smith-Hicks *et al.* 2010). However, the fact that Arc is strongly induced by LTP stimulation and accumulates in dendrites that are receiving high-frequency inputs is inconsistent with its synapse-weakening function (Link *et al.* 1995; Steward *et al.* 1998; Moga *et al.* 2004; Messaoudi *et al.* 2007). Okuno *et al.* (2012) provided an explanation of this contradiction by demonstrating that Arc modulates synapse specificity of LTP by weakening inactive synapses. Similarly to proteasomes, Arc is synaptically translocated when neurons are activated. But unlike proteasomes, which are recruited to activated spines by CaMKIIα, Arc is targeted to inactive synapses and removes surface GluA1 AMPA receptor subunits as evidenced by an inverse correlation between the amount of Arc and surface GluA1. Interestingly, the CaMKIIβ T287 to alanine mutant shows comparable amounts of binding to wild type, while the T287 to aspartate mutation greatly reduces Arc binding. In addition, CaMKIIβ–Arc interaction (both wild type and T287A) is disrupted by Ca^{2+}/CaM, indicating that the closed conformation of CaMKIIβ is important for the interaction with Arc. This 'inverse tagging' of an inactive synapse seems to play a key role in differentiating a 'strongly activated' synapse from a 'weakly activated' synapse, thus preventing over-excitation of local circuits by removing weakly activated synapses, and contrasting the synaptic strength of two synapses over time, thereby functioning as a homeostatic synaptic plasticity mechanism.

Casein kinase 2

Casein kinase 2 (CK2) is a serine/threonine kinase whose neuronal function is still under investigation, but is potentially important for a range of brain functions including learning and memory (Blanquet 2000). It is known that CaMKII recruits CK2 to NMDA receptors by forming a tripartite complex with GluN2B (Sanz-Clemente *et al.* 2010, 2013). In this complex, CK2 phosphorylates S1480 of GluN2B within the carboxyl terminal of the PDZ domain binding motif. Thus, this phosphorylation disrupts the interaction between GluN2B and PSD-95, leading to a reduction in the surface expression of GluN2B. Considering CK2 itself is constitutively active, this complex formation serves as a mechanism of activity regulation by increasing

the proximity between enzyme and substrate molecules. When the CaMKII–GluN2B interaction is disrupted, S1480 phosphorylation decreases and GluN2B surface level increases, indicating that the regulation of the physical distance between CK2 and GluN2B by CaMKII is a dominant factor in regulating the S1480 phosphorylation level of GluN2B. Similar to proteasome translocation, recruitment of CK2 also requires CaMKII activation (by Ca^{2+}/CaM and T286 phosphorylation) and GluN2B binding. CK2-mediated GluN2B phosphorylation seems to play an important role as an activity-dependent switch from GluN2B to GluN2A during development and LTP induction (Paoletti *et al.* 2013).

Calmodulin

CaMKII also functions in transcription. It has been well accepted that neuronal activity-dependent phosphorylation and activation of cAMP-response element-binding protein (CREB) are critical for LTP (Bourtchuladze *et al.* 1994). One of the CREB kinases is CaMKIV, which localizes mainly in the nucleus (Bito *et al.* 1996; Wayman *et al.* 2008). However, it is not clear where nuclear CaM comes from in neurons. Tsien and colleagues reported that the γ isoform of CaMKII plays a role in CREB-dependent transcription (Ma *et al.* 2014). CaMKIIγ-mediated CaM shuttling into the nucleus depends on transphosphorylation of T287 by CaMKIIβ, but not on the catalytic activity of CaMKIIγ itself. Thus, CaM trapping of CaMKII contributes to LTP not only at the synapse, but also in the nucleus, though by different mechanisms.

SynGAP

Synaptic GTPase-activating protein (SynGAP), a GAP of Ras and Rap (which in turn inactivates them), makes a complex with CaMKII through multiple PDZ domain protein 1 in dormant neurons where intracellular Ca^{2+} levels are low (Krapivinsky *et al.* 2004). In this state, SynGAP activity is inhibited by CaMKII-mediated phosphorylation, thus disinhibiting Rap and downstream p38 MAP kinase. This maintains low levels of synaptic transmission because of the removal of AMPA receptor from the synapse by Rap-p38 MAP kinase signaling (Zhu *et al.* 2002). CaMKII activation by NMDA receptor activation dissociates CaMKII from the multiple PDZ domain protein 1–SynGAP complex, leading to dephosphorylation and activation of SynGAP. Accordingly, inhibition of Rap-p38 MAP kinase signaling inhibits removal of AMPA receptor, which results in AMPA receptor accumulation at the synapse during LTP (Krapivinsky *et al.* 2004). The exact mechanism by which CaMKII mediates the phosphorylation of SynGAP under basal conditions is unclear. NMDA receptor-bound CaMKII may persistently exist in the hippocampus under basal conditions (Leonard *et al.* 1999; Bayer *et al.* 2006). Alternatively, unidentified kinases downstream of CaMKII may also participate.

However, it should be noted that the effect of SynGAP on synaptic strength and its regulation in relation to CaMKII are not simple and sometimes appear contradictory. There are other reports indicating that activity-dependent CaMKII activation phosphorylates SynGAP and this increases GAP activity (Oh *et al.* 2004; Carlisle *et al.* 2008), which suggest a negative effect of CaMKII-mediated phosphorylation of SynGAP in LTP. In contrast, Araki *et al.* (2015) recently showed that SynGAP phosphorylation by CaMKII contributes to LTP by removing SynGAP from stimulated spines, thus promoting AMPA receptor accumulation through Ras activation. Also, although previous studies suggest inhibitory roles of SynGAP on synaptic strength and AMPA receptor incorporation (Vazquez *et al.* 2004; Rumbaugh *et al.* 2006), a subsequent report demonstrates isoform-specific roles of SynGAP on synaptic strength (McMahon *et al.* 2012). SynGAP is a complex protein family containing variations in both amino and carboxyl termini. The difference in amino terminal sequences derives from alternative promoter usage which is regulated by synaptic activity and developmental stage, while variation in the carboxyl terminus is because of alternative splicing and is not regulated by synaptic activity. Over-expression of different isoforms resulting from various combinations of amino and carboxyl termini has different effects on synaptic strength judged by the amplitude and frequency of mEPSCs (McMahon *et al.* 2012). Moreover, not only CaMKII but also Cdk5 whose translocation to the PSD is mediated by CaMKII regulates the activity of SynGAP toward Ras and Rap differentially (Walkup *et al.* 2015). Thus, CaMKII-mediated regulation of SynGAP activity is more complex than initially thought and needs further investigation.

Microtubules

The role of microtubules in regulating synaptic plasticity has attracted less attention than actin given that microtubules mostly localize to the dendritic shaft rather than within spines. However, accumulating evidence implies that microtubules may also have a direct contribution to synaptic plasticity (Gu *et al.* 2008; Hu *et al.* 2008; Jaworski *et al.* 2009; Kapitein *et al.* 2011; Merriam *et al.* 2011, 2013), and one such role seems to be related with the transport of cargo proteins into activated spines (Fig. 1, Circle 6). Recently, CaMKII was shown to interact with microtubules in an activity-dependent manner near stimulated spines (Lemieux *et al.* 2012), suggesting a potential role of CaMKII in modulating both actin and microtubule components of the cytoskeleton during synaptic plasticity. Besides microtubules, CaMKII also binds to and phosphorylates KIF17, a kinesin-2 family microtubule-based motor protein (Guillaud *et al.* 2008). KIF17 is a neuron-specific molecular motor which transports GluN2B-containing vesicles by direct interaction with Mint1 (Setou *et al.* 2000), and transgenic mice over-expressing KIF17 show enhanced

learning and memory (Wong *et al.* 2002). The interaction between KIF17 and Mint1 is disrupted by CaMKII-mediated phosphorylation, leading to the release of cargo (Guillaud *et al.* 2008). Interestingly, recent reports demonstrate that microtubules transiently invade stimulated spines in an activity-dependent manner (Hu *et al.* 2008; Jaworski *et al.* 2009; Merriam *et al.* 2011, 2013). Thus, it is likely that the KIF17–cargo complex is directly transported to active spines, where the cargo is subsequently released by KIF17 phosphorylation by activated CaMKII. Also, it is known that microtubules preferentially invade spines whose F-actin content has increased recently (Merriam *et al.* 2013). Therefore, the structural and enzymatic functions of CaMKII act cooperatively on microtubules during synaptic plasticity.

Conclusion

Since CaMKII was identified as the 50 kD major PSD protein, its abundance was a mystery. Earlier studies mostly focused on the regulatory mechanism of kinase activity and identification of substrate proteins, which still did not explain why the abundance exceeded most of its substrate. However, it is now clear that two functions of CaMKII, the intrinsic structural role and kinase activity, intersect with each other to exert regulatory mechanisms in LTP. There is still much remaining to be solved before we can fully understand the role of CaMKII in synaptic plasticity and other important functions of the brain. What is the role of the CaMKIIα subunit, which is more abundant than β subunit? What is the relative contribution of Ca^{2+}/calmodulin binding and autophosphorylation in activation of CaMKII in LTP? What is the basal activity of CaMKII *in situ*? What anchors CaMKII at the synapse and how does it translocate to synapse by synaptic activity? Also, what is the mechanism that converts transient activation of CaMKII enzymatic activity and resulting detachment from F-actin into persistent activation of small G protein, accumulation of various synaptic protein, and spine growth? We still lack important knowledge on the regulation and functions of this protein, and a deeper understanding of these unsolved questions will shed new light on learning and memory mechanisms in the brain.

Acknowledgments and conflict of interest disclosure

We thank Drs Lily Yu and Thomas Chater for comments on the manuscript. The authors' work introduced in this review was supported by a Grant-in-Aid for Scientific Research for Young Researchers (K.K.), RIKEN, NIH grant R01DA17310, Grant-in-Aid for Scientific Research (A), Grant-in-Aid for Scientific Research on Innovative Area 'Foundation of Synapse and Neurocircuit Pathology' from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Human Frontier Science Program, Guangdong Key International Visiting Program (Y.H.) and CIHR grant MOP 111220, Canada Research Chairs Program, Canada Foundation for Innovation (K.O.). Y.H. receives research fund from Takeda Pharmaceuticals Co. Ltd. and Fujitsu Laboratories.

All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

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