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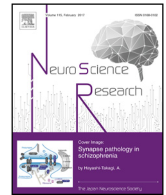

Review article

Molecular mechanism of hippocampal long-term potentiation – Towards multiscale understanding of learning and memory

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A R T I C L E I N F O

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Long-term potentiation (LTP) of synaptic transmission is considered to be a cellular counterpart of learning and memory. Activation of postsynaptic NMDA type glutamate receptor (NMDA-R) induces trafficking of AMPA type glutamate receptors (AMPA-R) and other proteins to the synapse in sequential fashion. At the same time, the dendritic spine expands for long-term and modulation of actin underlies this (structural LTP or sLTP). How these changes persist despite constant diffusion and turnover of the component proteins have been the central focus of the current LTP research. Signaling triggered by Ca^{2+}-influx via NMDA-R triggers kinase including Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). CaMKII can sustain longer-term biochemical signaling by forming a reciprocally-activating kinase-effector complex with its substrate proteins including Tiam1, thereby regulating persistence of the downstream signaling. Furthermore, activated CaMKII can condense at the synapse through the mechanism of liquid-liquid phase separation (LLPS). This increases the binding capacity at the synapse, thereby contributing to the maintenance of enlarged protein complexes. It may also serve as the synapse tag, which captures newly synthesized proteins.

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1. Introduction

In a book published in 1949, Donald Hebb described a model of how neurons in the brain store information (Hebb, 1949). He proposed that Ẅhen an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased̈. This property is now called Hebbian synaptic plasticity in honor of his conceptual establishment. However, it took more than 20 years to experimentally demonstrate the Hebbian plasticity in an animal. Bliss and colleagues found in rabbit dentate gyrus that a brief strong stimulation, or tetanic stimulation, of synaptic input (perforant fibers) persistently potentiates the subsequent synaptic transmission, a phenomena widely known as long-term potentiation or LTP (Bliss and Collingridge, 1993; Bliss and Gardner-Medwin, 1973; Bliss and Lømo, 1973; Nicoll, 2017). The same phenomena can be also induced *in vitro* in hippocampal slice preparation, which facilitated studies because of its accessibility (Alger and Teyler, 1976; Andersen et al., 1977; Lynch et al., 1976; Schwartzkroin and Wester, 1975; Yamamoto and Chujo, 1978) (Fig. 1). The molecular mechanism of LTP attracted the interest of a number of scientists. The long-standing question in the field is what is the nature and identity of "some growth process or metabolic change" that Hebb predicted. It can be rephrased as how a transient activation of a synapse triggers the persistent change in subsequent synaptic transmission efficacy. In this review, I start with the history of LTP research and then cover the recent results from my group as well as from others. Finally, I will conclude with what is required to understand the mechanism of learning and memory in the multiscale brain.

2. First step of LTP: induction

LTP is often considered in three steps: "induction", "expression" and "maintenance". The "induction" is a cellular signaling process that can be directly triggered by tetanic stimulation. The resultant signaling induces a change in the synapse that can be detected as an increase in the synaptic transmission, which is called "expression". Once transmission is enhanced, the "maintenance" mechanism perpetuates the status despite diffusion and turnover of component molecules as well as any other processes that mediates reverse biochemical reactions, such as a phosphatase for a kinase reaction.

In the '70 s and '80 s, efforts were made to develop pharmacological reagents that selectively modulate glutamate-mediated synaptic transmission (Krogsgaard-Larsen and Hansen, 1992; Watkins and Collingridge, 1989). This led to the identification of two major classes of glutamate receptors in hippocampal synapses, AMPA and NMDA-Receptors (AMPA-R and NMDA-R), based on their specific agonists. These two types of receptors play different roles. The basal transmission is mediated by AMPA-R but not by NMDA-R (Collingridge et al., 1983; Muller et al., 1988). The tetanic stimulation activates NMDA-R and induces a transient influx of Ca^{2+} into the postsynaptic compartment through the receptor, which initiates the induction process leading to long-term changes in AMPA-R, but not in NMDA-R (Kauer et al., 1990; Muller et al., 1988). The postsynaptic Ca^{2+} is necessary and sufficient for the induction of LTP (Malenka et al., 1988, 1989), which trig-

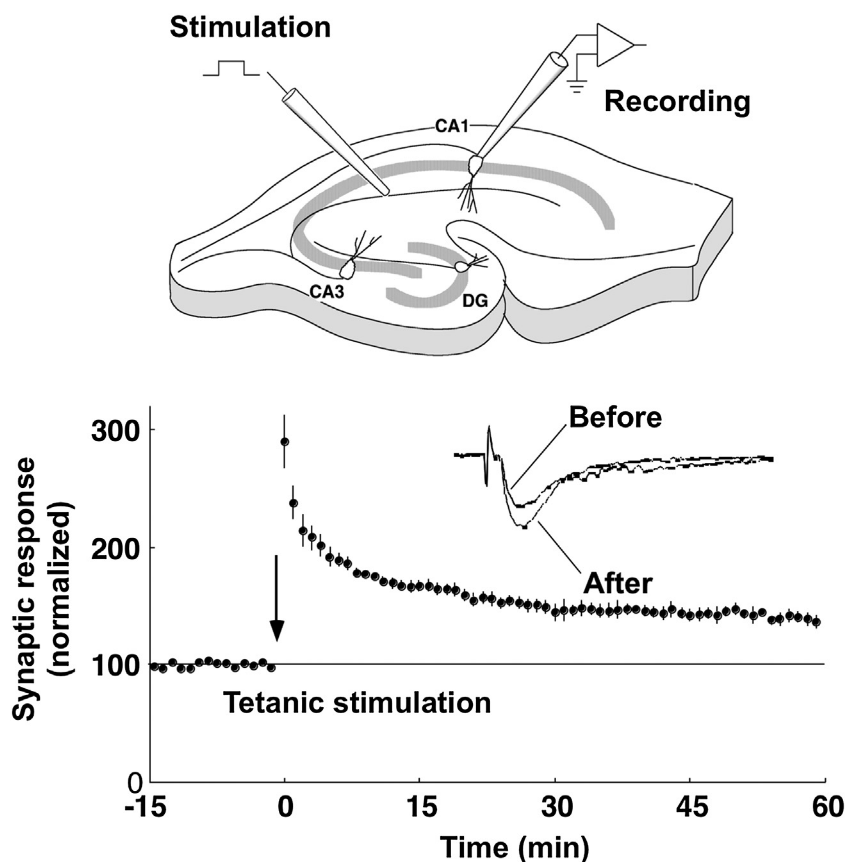

Fig. 1. Hippocampal long-term potentiation (LTP).
A typical experiment of LTP recording from hippocampal slice is shown. A brief strong stimulation, for example tetanic stimulation at 100 Hz for one second, potentiates the subsequent synaptic transmission for long-term. In slice, it lasts for a few hours. *In vivo*, it can last days.

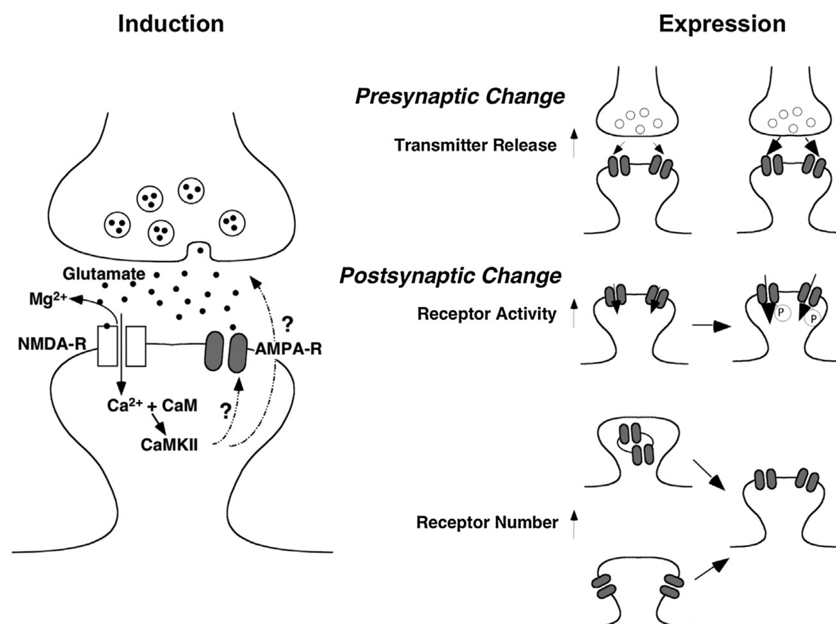

Fig. 2. Induction and different possibilities of expression mechanism of LTP.
Different models of molecular mechanism of LTP discussed in 90s. It was well accepted that during the induction of LTP, strong postsynaptic depolarization removes Mg^{2+} block of NMDA-R and let Ca^{2+} to flow into postsynaptic cytosol. This activates postsynaptic signaling molecules such as CaMKII (left). However, where it is expressed and maintained was long debated. While some argued an increase in presynaptic release probability, others insisted postsynaptic change, either by an increase in receptor activity or number (right).

gers the induction process leading to the enhancement of AMPA-R transmission.

3. Debate on pre- or postsynaptic expression and maintenance of LTP

However, as to the site of expression and maintenance, there was a great amount of confusion: whether it is presynaptic (an increase in transmitter release) or postsynaptic (an increase in postsynaptic sensitivity to glutamate) (Fig. 2). Using radiolabeled glutamate, it was demonstrated that induction of LTP increases the subsequent release of glutamate from hippocampal tissue (Dolphin et al., 1982). The failure of synaptic transmission, interpreted as the failure in synaptic vesicle release, was decreased after the induction of LTP, indicating a presynaptic increase in the probability of release (Stevens and Wang, 1994). The quantal analysis of the size of synaptic transmission also supports presynaptic expression (Bekkers and Stevens, 1990; Bolshakov and Siegelbaum, 1994; Malinow and Tsien, 1990). However, the same approach also led to different conclusions, with some proposing mixed pre- and postsynaptic changes or purely postsynaptic changes (Edwards, 1991; Kullmann and Nicoll, 1992; Larkman et al., 1992).

Other approaches also supported postsynaptic change. A selective increase in AMPA-R but not NMDA-R-mediated synaptic response is supportive of postsynaptic change rather than presynaptic change, which would affect both components equally (Foster and McNaughton, 1991; Kauer et al., 1988; Kullmann and Nicoll, 1992; Muller et al., 1988). An increase in responsiveness to exogenous glutamate (Davies et al., 1989) as well as an increase in the size of miniature excitatory postsynaptic current (mepsc) after LTP (Manabe et al., 1992) are suggestive of postsynaptic change. To add further complication, it turns out that a postsynaptically silent synapse, a synapse which does not have functional AMPA-R before the LTP induction, acquires AMPA-R response after the induction (unsilencing) (Isaac et al., 1995; Liao et al., 1995). This indicates that the decrease in the failure rate of synaptic response, which has been traditionally interpreted as a presynaptic increase in the probability of release, can also be attributed to a postsynaptic mechanism.

If the site of persistent change is presynaptic, because the initial induction of LTP requires postsynaptic NMDA-R activation and resulting Ca^{2+} influx, the postsynaptic side needs to somehow retrogradely communicate with the presynaptic side (Fig. 2). Several diffusible messengers have been proposed to play this role including nitric oxide, carbon monoxide, arachidonic acid, and platelet activating factor (Kato et al., 1994; O'Dell et al., 1991; Williams et al., 1989; Zhuo et al., 1993). However, the reproducibility of such studies was still questioned (Selig et al., 1996). Given these confusions, LTP studies obviously required a breakthrough.

4. AMPA-R trafficking

The limitation of these studies was that they relied on electrophysiological recording and statistical analyses of the size of synaptic response. The LTP studies required additional and independent readout. The development of several key technologies revolutionized the study of LTP. First, neuronal gene introduction techniques such as transgenic technologies, virus vectors, and chemical/physical methods allowed for specific molecular manipulation (gain-of-function such as overexpression or loss-of-function such as knockdown) or labeling (Haas et al., 2002; Klein et al., 1992; Malinow et al., 2010; Pettit et al., 1995). Second, discovery of GFP and its family of proteins allows for visualization of fine synaptic structures, detection of molecular dynamics, and even biochemical reactions underlying synaptic plasticity (Giepmans et al., 2006; Malinow et al., 2010; Miyawaki, 2005; Shaner et al., 2005; Yasuda, 2006). This is expedited by the employment of different fluorescence microscopy techniques, such as confocal, two-photon, and super-resolution microscopes (Choquet et al., 2021; Mainen et al., 1999).

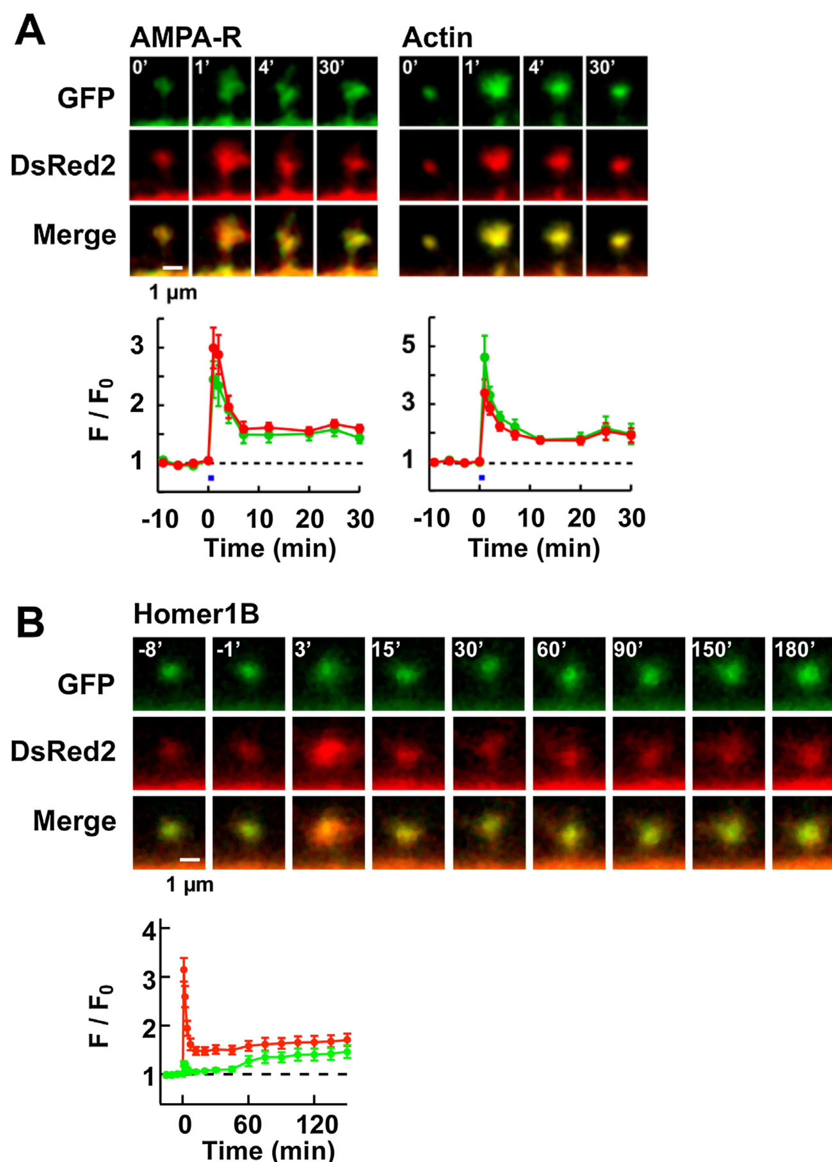

Fig. 3. Differential synaptic transport of proteins after LTP induction.
GluR1 subunit of AMPA-R, β-actin (A), and Homer1B (B) were each tagged with GFP (for AMPA-R, a pH sensitive GFP, super ecliptic pHluorine was used) and coexpressed with DsRed2 as a volume filler. LTP was induced by photo-uncaging of caged glutamate. Note that while GluR1 and actin were rapidly transported, Homer1B took one hour before it started accumulating at the synapse. From (Bosch et al., 2014).

A silent synapse, a synapse which does not show any AMPA-R response before LTP, can acquire response after LTP induction in an all-or-none fashion (Isaac et al., 1995; Liao et al., 1995). To explain such all-or-none appearance of the AMPA-R response, it was proposed that AMPA-Rs are translocated to the synapse upon induction of LTP. To visualize this process, GFP-tagged AMPA-R was expressed in neurons in hippocampal slice culture (Bosch et al., 2014; Shi et al., 1999). An LTP-inducing stimulation triggered the receptor trafficking to the synapse (Bosch et al., 2014; Shi et al., 1999) (Fig. 3). Both lateral diffusion and exocytosis of the intracellular pool of receptors take place in this process (Borgdorff and Choquet, 2002; Park et al., 2004; Passafaro et al., 2001; Patterson et al., 2010). Using a method to electrophysiologically tag exogenous AMPA-R, it was also demonstrated that induction of LTP triggers trafficking of AMPA-R to the synapse (Hayashi et al., 2000). These studies unambiguously showed that LTP is expressed and maintained postsynaptically, although still did not rule out the involvement of the presynaptic side.

5. Structural LTP

Excitatory synapses in central excitatory neurons are typically formed on dendritic spines, tiny mushroom-like protrusions on dendrites (Bosch and Hayashi, 2012; Harris and Kater, 1994; Hayashi and Majewska, 2005; Yuste, 2010). A single dendritic spine typically harbors one synapse on its head and connects to the dendritic shaft via a thin neck, thereby serving as a biochemical and electrical compartment. During development, the number and structure of dendritic spines gradually become mature. Immature neurons either do not have spines or have only filopodial structures lacking head (Hayashi and Majewska, 2005). It has been questioned how dendritic spines change their shape during neuronal activity and LTP.

Fifková et al. were the first to address this question. They analyzed the effect of tetanic stimulation on dendritic spine structure under an electron microscope in hippocampal dentate gyrus in intact animal that underwent LTP induction similarly to Bliss and

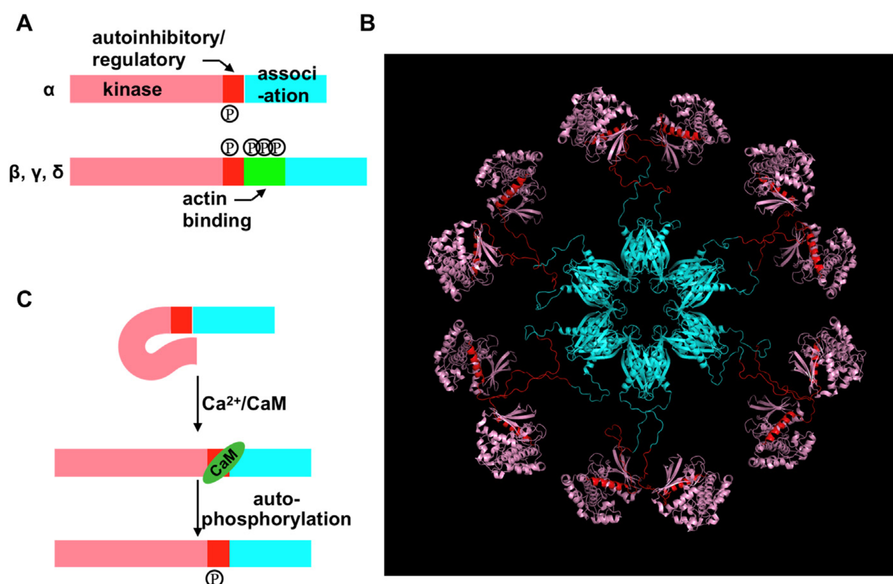

Fig. 4. Structure and regulation of CaMKII.
(A) Domain structure of CaMKII subunits. The major phosphorylation sites are indicated by P.
(B) 3D structure of the CaMKIIα holoenzyme modeled from cryo-EM images and crystal structures. Color code is consistent with the left, based on PDB Accession 5U6Y (Myers et al., 2017).
(C) Activation process of CaMKII. At basal status, the autoinhibitory domain inactivates the kinase domain. Ca^{2+}/CaM binding to the CaM-binding domain disinhibits the kinase. The autoinhibitory domain is phosphorylated, and the kinase becomes constitutively active in the absence of Ca^{2+}.

Lømo (1973). They found that dendritic spine starts expanding as early as 2 min, and lasted at least 23 h after tetanic stimulation (Fifková and Anderson, 1981; Fifková and Van Harreveld, 1977; Van Harreveld and Fifková, 1975). However, these observations were made across different preparations in fixed tissue.

Hosokawa et al. (1994) attempted a time-lapse imaging of dendritic spine in live neurons under LTP using DiI-labeling and a confocal microscope. They found an increase in the length of spines 3 h after chemical induction of LTP. Later, in a neuron expressing GFP, which fills the cytosol and reveals fine protrusive structures of a neuron, a local tetanic stimulation generates new spines and enlarges the existing spines (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Malinow et al., 2010; Okamoto et al., 2004).

However, in these studies, it was difficult to confirm whether the spine under observation underwent LTP or not, or if they were even stimulated, because presynaptic axons were not visible. This was resolved by the development of two-photon uncaging of caged-glutamate, which releases glutamate upon illumination with a two-photon laser. With this, it became possible to precisely stimulate a single spine and observe the processes underlying LTP, which could be also confirmed by electrophysiological recording (Matsuzaki et al., 2001, 2004). This confirms that LTP and synaptic trafficking of AMPA-R are accompanied by an expansion of dendritic spines, which I call here structural LTP or sLTP (Fig. 3). Like electrically induced and recorded LTP, sLTP is also persistent, lasting at least a few hours (Bosch et al., 2014; Kwon and Sabatini, 2011; Matsuzaki et al., 2004; Patterson et al., 2010). sLTP is specific to the stimulated spine and requires NMDA-R activation, recapitulating the properties of LTP. Therefore, it is highly likely that these two phenomena are mechanistically linked. By also using glutamate uncaging, correlated enlargement of presynaptic boutons was observed after sLTP induction (Meyer et al., 2014). The presynaptic boutons expand, but much more slowly compared with dendritic spines. The boutons took three hours to gradually expand whereas the spine immediately (~1 min) enlarges after stimulation. So, there is a disparity between the two sides of the synapse in the early phase of LTP, which is balanced at the later stage.

6. Trafficking occurs in a wide range of proteins in a regulated fashion

Extensive efforts have been made to identify the molecules involved in glutamatergic synaptic transmission. Both AMPA-R and NMDA-R subunits were cloned by employing a *Xenopus* oocyte cloning system and further by cross-hybridization (Hollmann et al., 1989; Moriyoshi et al., 1991; Traynelis et al., 2010). Proteins that directly and indirectly interact with the receptors were identified using various methods such as yeast-two hybrid screening, coimmunoprecipitation, and mass spectrometric analysis (Sheng and Hoogenraad, 2007). These efforts of more than two decades identified literally thousands of molecules of all different categories of cellular components, ranging from cell surface receptors and channels, scaffolding proteins, cytoskeletons, signal transduction machineries, protein synthesis, and membrane trafficking machineries. These proteins comprise an electron-dense structure beneath the synapse called the postsynaptic density (PSD).

When AMPA-R is trafficked and the dendritic spine expands, how do these PSD proteins behave? Are they translocated to the synapse together with AMPA-R or not? By using two-photon uncaging of caged-glutamate and GFP-fusion proteins, Bosch et al. (2014) systematically tested the translocation of different postsynaptic proteins (Fig. 3). They found that the translocation of proteins occurs in an ordered fashion. First, actin and its regulators are translocated within a few minutes in parallel with the spine expansion (Fig. 3A). By using Förster resonance energy transfer (FRET) imaging, it was found that actin is rapidly translocated within 20 s after LTP induction, which serves as a driving force to expand the dendritic spine (Okamoto et al., 2004). Then AMPA-R, actin binding proteins and kinases are translocated in a manner following the expansion of the dendritic spine. Finally, PSD scaffolding proteins such as Homer1B and Shank are translocated after one hour, in a manner requiring protein synthesis (Fig. 3B). This delayed translocation of PSD scaffolding proteins may be a mechanism of late phase LTP (L-LTP), which shares the requirement of protein synthesis (Bosch et al., 2014; Meyer et al., 2014; Panja and Bramham, 2014; Pinho et al., 2020) (Fig. 3B). This stepwise translocation of differ-

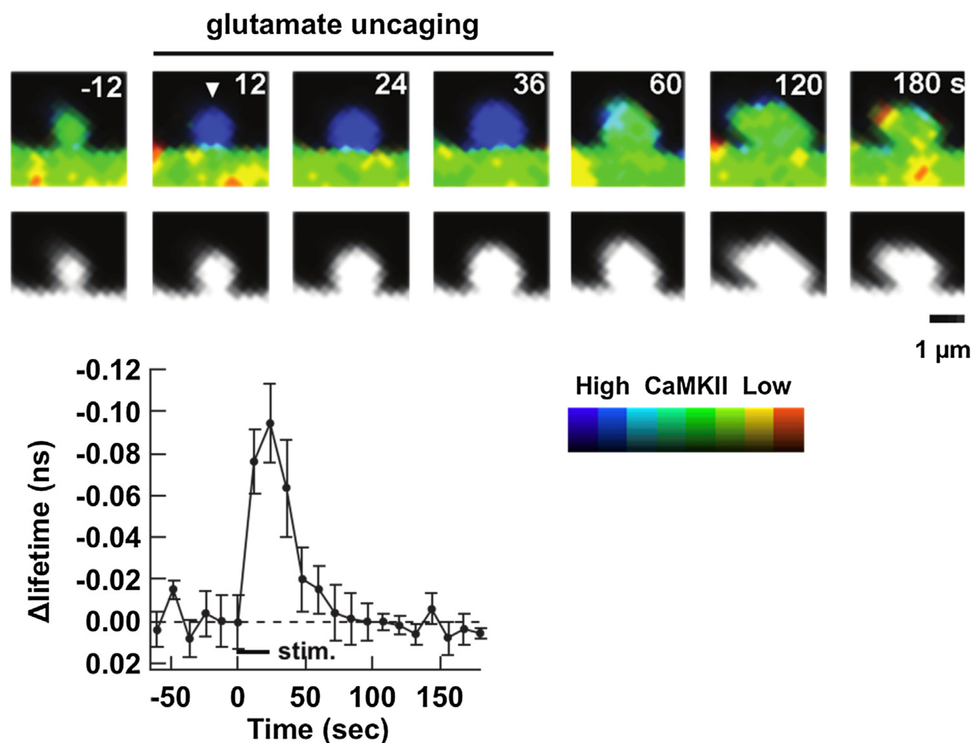

Fig. 5. Activation of CaMKII upon LTP induction observed by FRET.
CaMKII activity is transiently enhanced by stimulation by glutamate uncaging, but then quickly returned to the baseline levels. Note that blue in the pseudocolor indicates activation; modified from (Saneyoshi et al., 2019).

ent proteins explains the synaptic consolidation process where the synapse becomes more tolerant to reversal by depotentiating stimulation (prolonged, low frequency stimulation) after LTP induction over time. LTP can be more readily reversed by a depotentiation protocol if applied within a short time window after LTP induction when the synapse is still nascent (Fujii et al., 1991; Yang et al., 2008). As the potentiated state is gradually consolidated with the delayed translocation of proteins, synapses become more resistant to depotentiation.

7. Cellular signaling leading to LTP

Among literally hundreds of molecules implicated in LTP (Sanes and Lichtman, 1999), arguably, CaMKII is the most well-studied as a signaling molecule mediating LTP (Hell, 2014; Lisman et al., 2012). The rise in postsynaptic Ca^{2+} concentration triggers activation of CaMKII. Postsynaptic inhibition of the kinase blocks LTP (Malenka et al., 1989; Malinow et al., 1988, 1989). Consistently, genetic ablation of CaMKII impairs LTP as well as learning and memory (Hinds et al., 1998; Silva et al., 1992). Also, introduction of the active form of CaMKII is sufficient to induce enhancement of synaptic transmission and synaptic insertion of AMPA-R (Hayashi et al., 2000; Lledo et al., 1995; Pettit et al., 1994; Poncer et al., 2002; Shirke and Malinow, 1997). Therefore, activity of CaMKII is necessary and sufficient to induce LTP.

CaMKII has an N-terminal kinase domain, an autoinhibitory/regulatory domain and a C-terminal association domain (Fig. 4A, B). In inactive CaMKII, the autoinhibitory domain masks the kinase domain. Ca^{2+}/calmodulin binding to the regulatory domain unmasks the inhibition of the catalytic domain and activates the kinase (Fig. 4C). Once activated, CaMKII not only phosphorylates various substrates but also autophosphorylates itself at threonine (T) 286, located in the autoinhibitory domain (Hanson et al., 1989; Miller et al., 1988; Schworer et al., 1988). Once this reaction takes place, the autoinhibitory domain no longer inhibits the catalytic domain, thereby making CaMKII constitutively active.

Different models have been proposed as to CaMKII's regulation and involvement in the maintenance of LTP. An earlier model proposed that the constitutive, Ca^{2+}-independent activity of CaMKII increases persistently after LTP induction, thereby maintaining the enhanced synaptic transmission (Fukunaga et al., 1993; Lisman et al., 2002). Lee et al. challenged this view by optically monitoring the CaMKII activity by using a FRET-FLIM sensor, Camui (Kwok et al., 2008; Takao et al., 2005), during sLTP. They found that the duration of CaMKII activation lasts only ~1 min after the stimulation (Chang et al., 2017; Lee et al., 2009; Saneyoshi et al., 2019) (Fig. 5). The same group generated a photoactivatable inhibitor of CaMKII and found that photoactivation of the inhibitor is effective in blocking the structural plasticity only during induction but not during maintenance (Murakoshi et al., 2017).

However, the amount of total CaMKII at the synapse increases after LTP induction persistently (Bosch et al., 2014; Shen and Meyer, 1999). Lee et al. (2009) found that CaMKII activity returns to the basal level but they did not confirm that the basal level of activity is null. Indeed, there is always basally activated CaMKII in unstimulated hippocampal tissue preparation (Fukunaga et al., 1993; Takao et al., 2005). These indicate that there is a net increase in the amount of active CaMKII at each synapse after LTP induction. Therefore, a consensus has not been yet met whether kinase activity is required during the maintenance phase of LTP.

8. Possible substrates of CaMKII

An obvious question is which CaMKII substrate is necessary for LTP. More than 400 phosphorylation sites of CaMKII are reported (Phosphosite Plus, https://www.phosphosite.org), many of which are relevant to the synaptic functions. To name a few, AMPA-R

subunit GluA1, NMDA-R subunits GluN2A and 2B, cell adhesion molecule Neuroligin, nitric oxide (NO) synthase, synaptic scaffolding protein Homer, Shank, PSD-95, and SynGAP, and a transcription factor CREB, are among known substrates. The phosphorylation of AMPA-R is an obvious interest as a mechanism of LTP (Barria et al., 1997b; Hayashi et al., 1997; McGlade-McCulloh et al., 1993; Raymond et al., 1993). The GluA1 subunit of AMPA-R is phosphorylated by CaMKII at serine 831 (S831, numbering based on mature protein after signal peptide cleavage), which is proposed to increase the channel conductance, thereby contributing the increased transmission (Barria et al., 1997a; Derkach et al., 1999; Diering et al., 2016; Roche et al., 1996). However, S831 does not conform to the known consensus sequence of CaMKII substrates, RXXS/T, and its affinity towards CaMKII is significantly lower than other substrates (Özden et al., 2020). Also, S831 phosphorylation is not required for CaMKII-induced trafficking of AMPA-R (Hayashi et al., 2000).

Hosokawa et al. used Phos-tag SDS-PAGE to detect the stoichiometry of GluA1 phosphorylation (Hosokawa et al., 2015; Kinoshita et al., 2006). Phos-tag specifically interacts with phosphorylated residues and slows down the migration of phosphorylated protein in SDS-PAGE compared with the unphosphorylated counterpart. By blotting the sample with an antibody that equally detects both phosphorylated and unphosphorylated proteins, one can estimate the stoichiometry of the phosphorylation (Hosokawa et al., 2010). By using this method, Hosokawa et al. (2015) found that the stoichiometry of S831 phosphorylation is less than 1% of total GluA1, even after induction of chemical LTP in dissociated culture. This is too low to explain the observed increase in synaptic transmission. Antibodies against phosphorylated AMPA-R at S831 and S845 (a protein kinase A phosphorylation site) have been widely used to study the significance of the AMPA-R phosphorylation (Mammen et al., 1997) but these antibodies are highly sensitive and ironically detected trace amount of phosphorylation, which may or may not be functional. On the other hand, a double knock-in animal of S831A and S845A indeed shows impairment in LTP and long-term retention of memory (Lee et al., 2003). Because S831 and S845 phosphorylation is higher in younger tissue (Hosokawa et al., 2015), it might be due to a developmental effect. Therefore, phosphorylation of AMPA-R may occur *in vivo* and have a functional role but not it is not the way initially thought.

9. Cytoskeletal role of CaMKII

CaMKII has been considered as a molecule that mediates Ca^{2+} signaling. However, there are a few mysteries about CaMKII if it is purely a signaling molecule. First, CaMKII is highly abundant in the brain, especially at the synapse, comprising approximately 10–30% of the total protein in the hippocampal PSD fraction (Erondu and Kennedy, 1985). If CaMKII is a kinase involved in signaling, it is not required in such abundance, as a single molecule of CaMKII can phosphorylate multiple substrate molecules. Second, CaMKII forms a dodecamer or tetradecamer in rotational symmetry through its association domain, as revealed by electron microscopic observation and X-ray crystallography (Hoelz et al., 2003; Kanaseki et al., 1991; Myers et al., 2017). The association domain shares structural similarity with a protein of unknown function found in green algae and bacteria (McSpadden et al., 2019). During the evolution of animals, the common ancestor of CaM family proteins (CaMKII as well as CaMKI, CaMKIV, and CaMKK) fused with the association domain to form the unique structure of CaMKII, which is conserved in all living animal lineages. However, the functional significance of such oligomeric structure has not been made entirely clear.

CaMKII is encoded by four different genes CAMK2A-D, each translated into CaMKIIα-δ subunits (Tobimatsu and Fujisawa, 1989). All subunits are expressed in the brain but CaMKIIα and β heterooligomers are the dominant subunits in the forebrain. They share a basic structure except for the linker sequences between the autoinhibitory/regulatory region and the association domain. CaMKIIα subunit has a short linker whereas β subunit has a longer linker. Importantly, CaMKIIβ subunits can interact with filamentous actin (F-actin) through this linker region (Lin and Redmond, 2009; O'Leary et al., 2006; Shen et al., 1998). Due to its oligomeric structure, a single CaMKII heteromer containing CaMKIIβ can interact with more than one filament and bundle them together. With this mechanism, CaMKII may serve as a structural element (Fink et al., 2003; Okamoto et al., 2007). Consistently, down-regulation of the CaMKIIβ subunit, but not α subunit using shRNA converts the dendritic spine structure into a filopodial structure (Okamoto et al., 2007). This phenotype could be rescued by overexpression of CaMKIIβ with a kinase null mutation, indicating that the structural role of CaMKIIβ is independent of the kinase activity. Then how does kinase activity affect the structural role? The linker region is intrinsically disordered with multiple autophosphorylation sites and autophosphorylated CaMKIIβ dissociates from F-actin (Kim et al., 2015). With these phosphorylations, the induction of LTP unbundles F-actin, thereby generating a transient window for F-actin remodeling by actin regulators such as Arp2/3 and cofilin (Kim et al., 2015). In this way, CaMKII stabilizes synaptic F-actin by bundling them together in a naïve synapse but allows for a transient time-window of actin modification after LTP-inducing stimulation.

10. Reciprocal activation between CaMKII and effector proteins

Then what is the role of the α subunit of CaMKII, which is more abundant than β? CaMKII is known to interact with the intracellular carboxyl tail of NMDA-R subunit GluN2B through a binding pocket called the T-site, which is usually occupied by the autoinhibitory domain (Bayer et al., 2001). The same binding mode is shared by several other proteins including endogenous CaMKII inhibitory peptide CaMKIIN, a Rac guanine nucleotide exchange factor (RacGEF) TIAM1, GJD2/connexin 36, LRRC7/densin-180, *Drosophila* EAG (ether-à-go-go or KCNH) voltage-dependent potassium channel, and small G-protein Rem2 (Alev et al., 2008; Bayer et al., 2001; Chao et al., 2010; Royer et al., 2018; Saneyoshi et al., 2019; Sun et al., 2004; Vest et al., 2007; Walikonis et al., 2001). This binding is triggered by Ca^{2+}/calmodulin and once bound, persists even after chelation of Ca^{2+} (Bayer et al., 2001; Hosokawa et al., 2021; Saneyoshi et al., 2019). While GluN2B and EAG potassium channels has canonical CaMKII phosphorylation site R-X-X-S/T sequence, others are pseudosubstrates, having non-phosphorylatable residues instead of S/T (Özden et al., 2020). Interestingly, this binding locks CaMKII into an active conformation (Bayer et al., 2001; Saneyoshi et al., 2019). Tiam1, in turn, is phosphorylated and activated by CaMKII, through this interaction. Thus, CaMKII and Tiam1 form a reciprocally activating kinase-effector complex (RAKEC). The formation of RAKEC with Tiam1 is constitutive, lasting at least 30 min after LTP induction. In this way, RAKEC may be a mechanism to maintain CaMKII activity and, at the same time, specific downstream signaling thereby contributing to the maintenance of LTP.

11. Liquid-liquid phase separation of CaMKII as a novel mechanism of LTP

In order to explore CaMKII-associated protein (CaMKIIAP) more systematically, a pull-down experiment was carried out from brain tissue. The mass spectrometric analysis identified >100 proteins (Baucum et al., 2015), though whether the binding involves the T-site or not is not tested in this study. I came to an idea that CaMKII

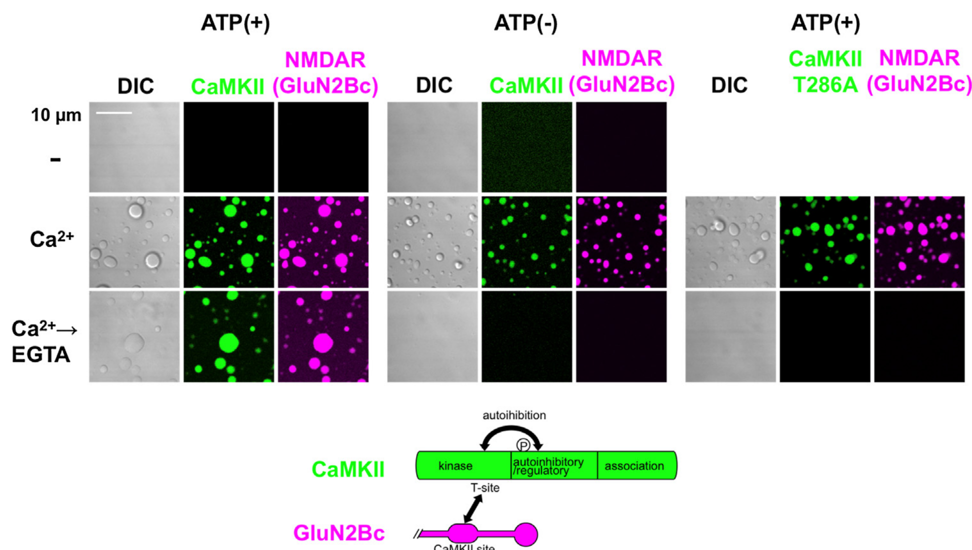

Fig. 6. Liquid-liquid phase separation of CaMKII and GluN2B.
CaMKII and GluN2B carboxyl terminus (GluN2Bc) were purified. GluN2Bc was labeled with a dimeric fluorescent protein to match the actual receptor stoichiometry. CaMKII, GluN2Bc, and calmodulin did not undergo liquid-liquid phase separation in the absence of Ca^{2+}, but they were condensed by the addition of Ca^{2+}. The condensate persisted even after the chelation of Ca^{2+} with EGTA. This requires T286 phosphorylation because in the absence of ATP or by introducing the T286A mutation to CaMKII, the condensate did not persist after EGTA addition. From (Hosokawa et al., 2021).

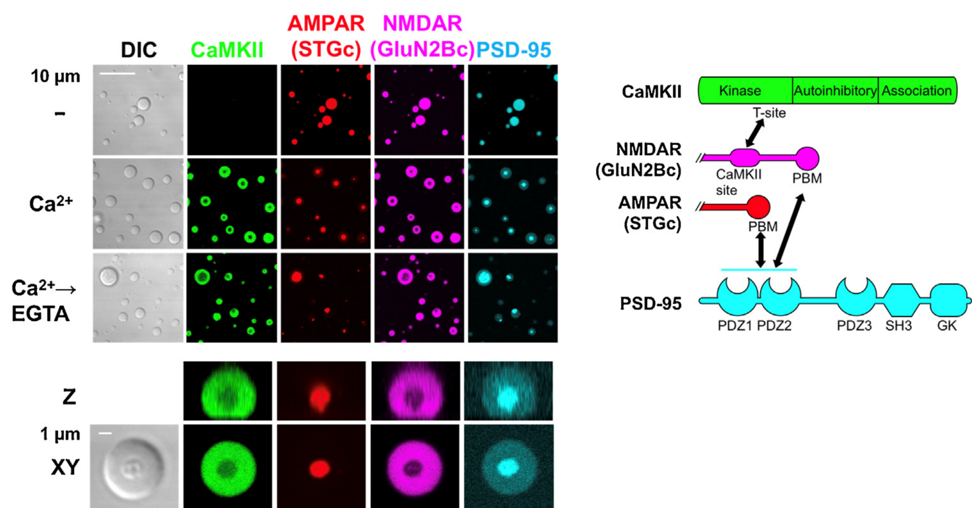

Fig. 7. Separation of AMPA-R and NMDA-R by CaMKII liquid-liquid phase separation.
In the absence of Ca^{2+}, AMPA-R (represented by Stargazin (STGc), NMDA-R (represented by carboxyl tail of GluN2B subunit (GluN2Bc)), and PSD-95 are phase-separated, while CaMKII was present in the dilute phase. Upon addition of Ca^{2+}, STGc, and PSD-95 formed an in-phase phase and were separated from GluN2B and CaMKII. This persisted after the chelation of Ca^{2+} with EGTA From (Hosokawa et al., 2021).

may serve as a crosslinker of the proteins thereby accumulating them at the synapse. Especially, I was intrigued by a phenomenon of liquid-liquid phase separation (LLPS) of biological macromolecules, where macromolecules such as RNA or proteins spontaneously condensate and form a protein droplet in solvent. The proteins that undergo LLPS often have multimeric structure, weak interaction, and an intrinsically disordered region (Banani et al., 2017; Feng et al., 2019; Hayashi et al., 2021; Hyman et al., 2014; Shin and Brangwynne, 2017). The multimeric structure of CaMKII is indeed an ideal structure to undergo LLPS with multiple substrates and pseudosubstrate proteins, which themselves can be oligomers and cross-link them together.

Purified CaMKII and GluN2B carboxyl tail were mixed and observed under microscope to test if they undergo LLPS. In the absence of Ca^{2+}, these two proteins remained in the diluted phase and no condensate was observed. However, when Ca^{2+}/calmodulin was added, they formed protein droplets (Fig. 6). Once formed, the droplets persist even after the addition of EGTA, in a manner dependent on the autophosphorylation at T286. Therefore, CaMKII can remember the transient Ca^{2+} signal as a form of LLPS. Whether CaMKII undergoes LLPS *in vivo* is not fully demonstrated, but rapid and constant turnover of synaptically accumulated CaMKII as revealed by fluorescence recovery after photobleaching assay (FRAP) or photoactivatable fluorescence protein is consistent with this view (Bosch et al., 2014; Lu et al., 2014; Okamoto et al., 2004).

Addition of more proteins to the system revealed that activated CaMKII not only undergoes LLPS by itself but also defines a postsynaptic nanodomain through this mechanism (Hosokawa et al., 2021). When AMPA-R (represented by an auxiliary subunit Stargazin), NMDA-R, and PSD-95 are present in the system, activation of CaMKII partitioned AMPA-R and NMDA-R into two different phases (Hosokawa et al., 2021) (Fig. 7). Consistently at a synapse,

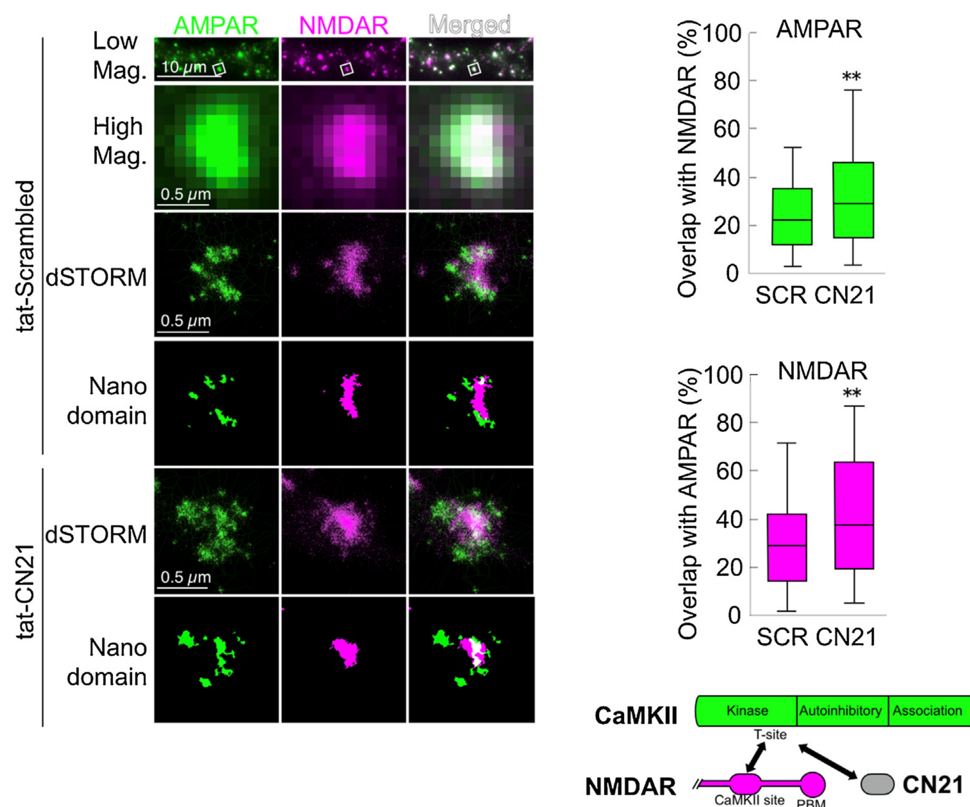

Fig. 8. Segregation of AMPA-R and NMDA-R into nanodomain by CaMKII.
Nanodomains of the AMPA and NMDA-Receptors were visualized by super-resolution microscopy dSTORM. Inhibition of the interaction between CaMKII and NMDA-Receptor GluN2B with CN21 reduced the separation of nanodomains. From (Hosokawa et al., 2021). SCR: scrambled peptide.

CaMKII segregates AMPA-R and NMDA-R into different subsynaptic nanodomains through this mechanism as revealed by superresolution microscopy (Choquet et al., 2021; Hosokawa et al., 2021) (Fig. 8). The same mechanism places AMPA-R beneath the synaptic vesicle release site, thereby forming a synaptic nanocolumn, a vertical arrangement of presynaptic transmitter release site and postsynaptic glutamate receptor (Hruska et al., 2018; Liu et al., 2021; Tang et al., 2016). Because AMPA-R is not saturated with glutamate at the synapse (Liu et al., 1999; Patneau and Mayer, 1990; Tong and Jahr, 1994; Xie et al., 1997), this might serve as a novel mechanism of LTP mediated by CaMKII (Fig. 9).

Also, the formation of the condensed phase of CaMKII can serve as a synapse tag, a conceptual binding site specifically formed in a synapse that underwent LTP for newly synthesized protein (Frey and Morris, 1997; Pinho et al., 2020; Redondo and Morris, 2011). A tag should be formed at a synapse without requiring protein synthesis and serves as a binding site for newly synthesized proteins. CaMKII can interact with multiple proteins through its T-site as well as indirectly through F-actin, which also binds multiple proteins (Okamoto et al., 2009). Therefore, CaMKII condensed at the synapse by LLPS mechanism may satisfy the long-sought identity of a synapse tag.

12. Synaptic plasticity and human diseases

It has been known that phencyclidine, an NMDA-R channel blocker, induces a schizophrenic behavior in human (Javitt, 1987). Also, a rodent model of NMDA-R hypofunction exhibits an abnormal social behavior, similar to the negative symptoms of schizophrenia (Mohn et al., 1999). Recent advances in exome sequencing led to the discovery of genetic mutations in synaptic proteins including NMDA-R and its binding partners including CaMKII, as well as synaptic adhesion molecules such as postsynaptic neuroligin and its presynaptic counterpart, neurexin in human patients with disorders such as schizophrenia, autism, intellectual abnormality, and attention deficit hyperactivity disorder (ADHD) (Obi-Nagata et al., 2019; XiangWei et al., 2018). Of a particular interest to me is the F98S mutation of CaMKII α subunit, which causes intellectual disability in patients (Küry et al., 2017). F98 was identified by Bayer et al. as an amino acid residue CaMKII α subunit that constitutes the T-site and is important for binding to GluN2B (Bayer et al., 2006). This mutation is expected to disrupts LLPS, suggesting requirement of LLPS of CaMKII in normal cognitive functions. Not just genetic mutations, autoantibody against NMDA-R causes acute psychosis, especially young females carrying teratoma (Dalmau et al., 2007). Repeated stress in rodents reduces LTP and impairs AMPA-R trafficking which leads to memory deficit (Yuen et al., 2012). The expression of GluA1 subunit of AMPA-R and CaMKII are downregulated in patients with major depressive disorder (Fuchsova et al., 2015; Tochigi et al., 2007; Duric et al., 2013). These studies indicate that the abnormalities in excitatory synaptic transmission and plasticity might cause a wide variety of neuropsychiatric and neurocognitive disorders and paved a way to development of novel therapeutic approaches towards these disorders.

13. Concluding remarks

Already in 1991, it was ridiculed that LTP is a long-term problem (Edwards, 1991). In 1999, Sanes and Lichtman wrote a review titled "Can molecules explain long-term potentiation?" (Sanes and Lichtman, 1999). This well represents the sentiment of the field at that time with an endless debate on pre and post. However, it was the year when LTP studies with novel approaches (Shi et al., 1999)

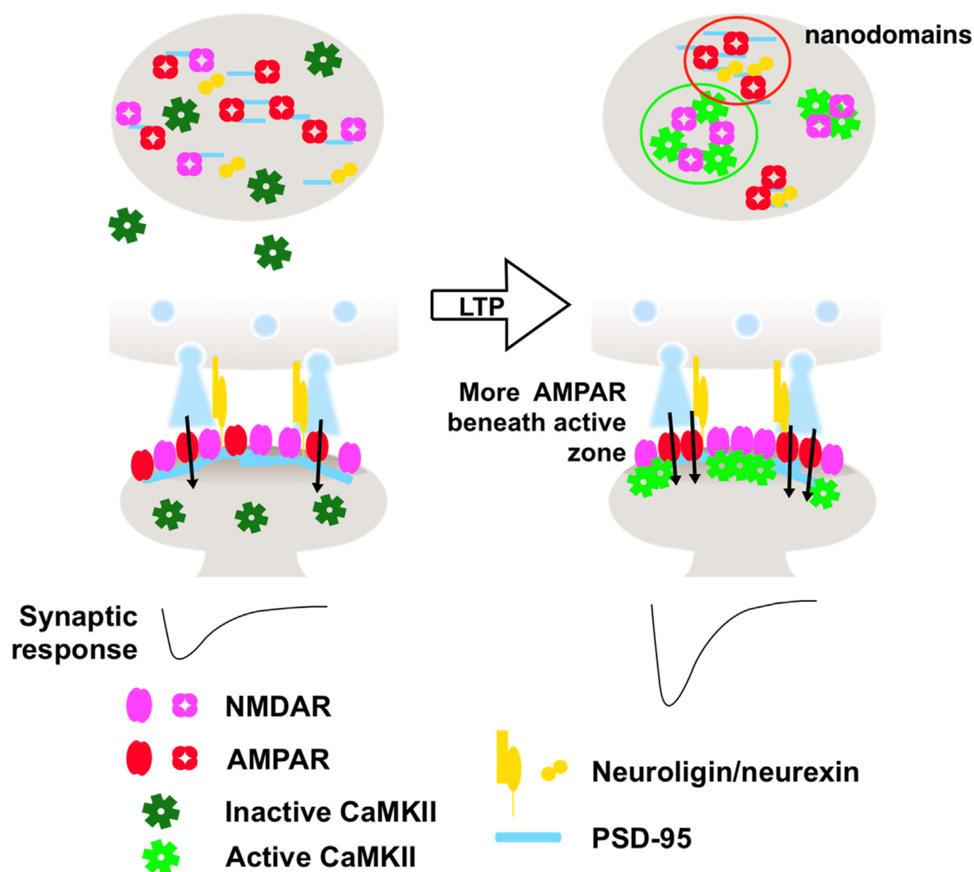

Fig. 9. Alignment of presynaptic release site and postsynaptic AMPA-R by CaMKII as a novel mechanism of synaptic plasticity.
CaMKII segregates AMPA-R and NMDA-R into nanodomains. More AMPA-R are concentrated beneath the transmitter release site, resulting in more efficient synaptic response. This may be a new mechanism of synaptic plasticity. From (Hosokawa et al., 2021).

started emerging and continues to this day. Indeed, it has been still a long-term journey, but I trust ongoing works will elucidate the mystery of memory, the biggest remaining question for mankind. Especially, a multiscale understanding from molecule to circuit is crucial. Elucidating when and where LTP takes place in living animal during the formation and consolidation of memory will be a next major challenge of LTP studies. Application of technologies that allowed us to optically manipulate synaptic plasticity described based on molecular mechanisms here will provide versatile information on this point (Hayashi-Takagi et al., 2015; Murakoshi et al., 2017).

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

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