



Regulation of synaptic nanodomain by liquid–liquid phase separation: A novel mechanism of synaptic plasticity

Pin-Wu Liu, Tomohisa Hosokawa¹ and Yasunori Hayashi

Abstract

Advances in microscopy techniques have revealed the details of synaptic nanodomains as defined by the segregation of specific molecules on or beneath both presynaptic and postsynaptic membranes. However, it is yet to be clarified how such segregation is accomplished without demarcating membrane and how nanodomains respond to the neuronal activity. It was recently discovered that proteins at the synapse undergo liquid–liquid phase separation (LLPS), which not only contributes to the accumulation of synaptic proteins but also to further segregating the proteins into subdomains by forming phase-in-phase structures. More specifically, CaMKII, a postsynaptic multifunctional kinase that serves as a signaling molecule, acts as a synaptic cross-linker which segregates certain molecules through LLPS in a manner triggered by Ca^{2+} . Nanodomain formation contributes to the establishment of trans-synaptic nanocolumns, which may be involved in the optimization of spatial arrangement of the transmitter release site and receptor, thereby serving as a new mechanism of synaptic plasticity.

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Synaptic transmission, synaptic plasticity, Liquid–liquid phase separation, Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), Glutamate receptor, Synaptic adhesion molecules, Synaptic nanodomain, Trans-synaptic nanocolumn.

Introduction

A synapse is not a homogeneous structure. They have discrete functional nanodomains. Synaptic vesicles are segregated at specialized structures at the presynaptic membrane called the active zone, where specific proteins that tether the vesicles are accumulated, thereby making the vesicles ready for exocytosis [1,2]. The synaptic membrane next to the active zone is enriched with voltage-gated Ca^{2+} channels to generate Ca^{2+} nanodomains upon arrival of an action potential to the presynaptic terminus [3]. Postsynaptically, different types of glutamate receptors are segregated into distinct nanodomains [4–6]. Furthermore, these receptors and downstream signaling molecules accumulate and form the postsynaptic density (PSD), an electron dense disc-like structure beneath the membrane. The PSD itself also has structural heterogeneity observed as perforations and notches, where the electron density is significantly lower than the rest [7]. These synaptic nanodomains on both sides of a synapse undergo coordinated regulation by neuronal activation [8–10].

Most intriguingly, these segregations are accomplished without any demarcating membrane that confines the molecules. This is unlike other membrane-bound organelles, such as the endoplasmic reticulum and lysosomes. Nevertheless, the molecules within the PSD undergo constant turnover between the cytosol or even with adjacent synapses while the identity of a synapse is maintained over a period of months [11–16]. Even though these contradictory properties of synaptic proteins are both critical for synaptic functions [17], how it is accomplished and how it is regulated remained largely unclear. Recently, a new concept of biological liquid–liquid phase separation (LLPS) was introduced in the field of neuroscience [18–21], which reasonably explains the accumulation of molecules, as well as the segregation inside of these protein condensates. In this review, we will describe how LLPS can explain accumulation and further segregation of synaptic component proteins, with an emphasis on a new role of postsynaptic Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) in LLPS.

Nanodomain observation by super-resolution imaging

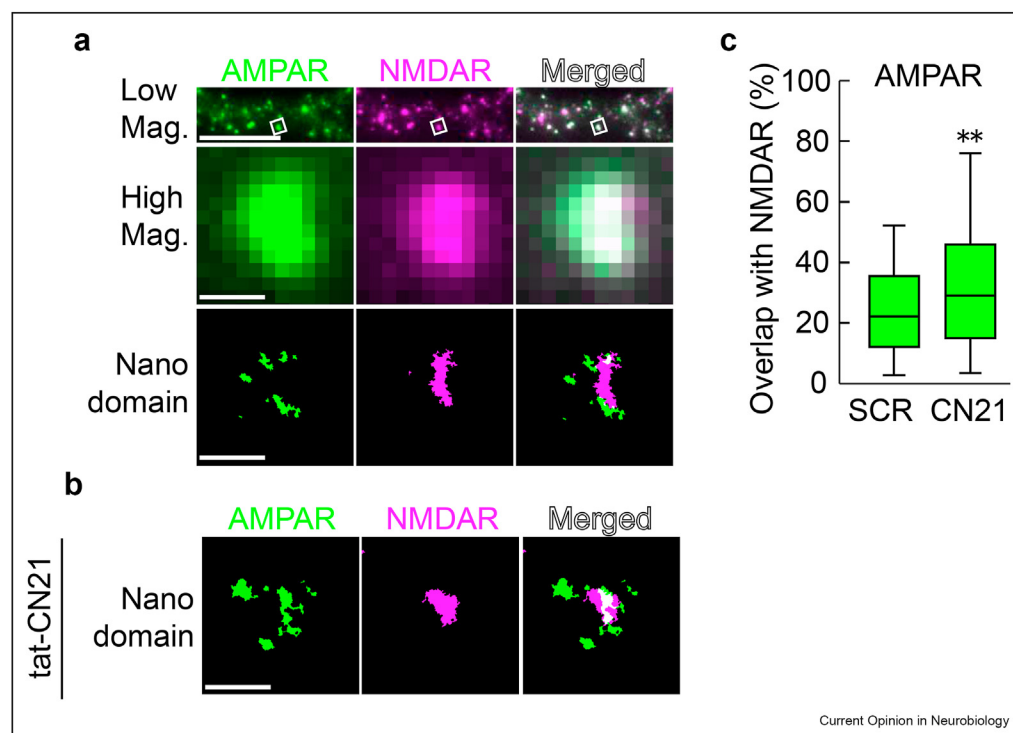
The size of a typical excitatory synaptic contact in the mammalian central nervous system is ~ 500 nm or less in diameter [22]. This is close to the resolution limit of conventional light microscopes and therefore it was not possible to observe the internal structures of a synapse. However, with the development of novel super-resolution microscopy techniques, such as direct stochastic optical reconstruction microscopy (dSTORM), stimulated emission depletion microscopy (STED), universal point accumulation in nanoscale topography (uPAINT) and single particle tracking photoactivation localization microscopy (spt-PALM), we can now observe the internal structures of a synapse.

These studies revealed that AMPA-type glutamate receptors (AMPA) form clusters on the postsynaptic membrane [5,23,24] (Figure 1A). The number of clusters in a synapse ranges from 0 to 6 (average of 1.5–2.5 clusters depending on the microscopy method used) in the case of hippocampal neurons. The size of a single AMPAR nanodomain is typically around 70–80 nm, each of which contains 20–25 AMPARs. The AMPARs inside the nanodomain are largely immobile, whereas those outside are freely diffusing. Furthermore, AMPARs show

high mobility at the synapse when the synapse is unstimulated. However, once the Ca^{2+} concentration in the synapse is increased, the movement of AMPARs is restricted [25,26].

A clustered distribution of NMDA-type glutamate receptors (NMDARs) is also observed [4–6]. Although AMPA receptors show multiple nanodomains of similar size, NMDARs form one large nanodomain in the middle of the postsynaptic membrane (Figure 1A) [5,24]. The second largest NMDAR nanodomain is significantly smaller than the largest one [5]. Interestingly, AMPARs and NMDARs do not colocalize in the same cluster, rather segregate from each other (Figure 1A). The NMDAR nanodomain is surrounded by several AMPAR nanodomains [5,24]. A similar observation was made by using SDS-digested freeze-fracture replica labeling (SDS-FRL) electron microscopy in a cerebellar mossy fiber synapse [27]. The NMDAR nanodomain is further subdivided into GluN2A and GluN2B enriched domains [4,6]. However, the observation of climbing fiber-Purkinje cell and parallel fiber-interneuron synapses by SDS-FRL electron microscopy shows homogeneous distribution of NMDARs throughout the postsynaptic membrane, whereas mossy fiber-granule cell synapses show

Figure 1



STORM imaging for postsynaptic nanodomains. NMDAR and AMPAR on hippocampal neuron were observed by dSTORM. (a) Conventional epifluorescent images of dendrite (Low Mag.) and a synapse (High Mag.) did not reveal nanodomains whereas dSTORM images revealed that AMPAR and NMDAR are segregated with each other (nanodomain). (b) Treatment of cell-permeable tat-CN21 resulted in the increase of the overlapping of AMPAR-NMDAR nanodomains (tat-CN21). (c) Quantification of overlapping rate of AMPAR with NMDAR from (a) and (b). Scale bars, 10 μm (Low Mag). And 0.5 μm (High Mag. and nanodomain). AMPAR, AMPA-type glutamate receptors; NMDAR, NMDA-type glutamate receptors.

segregated clusters, indicating that the nanodomain formation is dependent on synapse type [27]. Subsynaptic localization of NMDARs is also regulated. The phosphorylation at Tyr-1472 of GluN2B has been proposed to regulate NMDAR localization as the Y1472F mutant affects localization at synapses in the amygdala [28]. In contrast to the ionotropic glutamate receptors, a metabotropic glutamate receptor subtype mGluR5 is homogeneously distributed possibly due to its rapid and constant lateral diffusion [5].

PSD as a segregated compartment of synaptic proteins from the cytosol

Then what is the mechanism that segregates different types of receptors on the synaptic surface? The glutamate receptors interact with synaptic scaffolding proteins through their intracellular region, which determines the distribution and the dynamics of the receptor molecules. These scaffolding molecules, together with various other proteins involved in synaptic signal transduction, comprise the PSD, an electron-dense structure beneath the synaptic membrane [22,29]. The localization of the PSD beneath the postsynaptic membrane makes it a perfect structure to orchestrate the synaptic signal transduction and structure. The constituent molecules of the PSD are condensed from the cytosol [30,31]. Fluorescent microscopy revealed that the PSD is not static, typically the case for pathological protein aggregates, rather is dynamic. This can be demonstrated by photobleaching GFP-tagged PSD components which shows recovery over time, suggesting spontaneous turnover and mixing of the components [12,13,29,32,33]. Furthermore, new component molecules are translocated and incorporated into the existing PSD during long-term potentiation (LTP), which eventually leads to an increase in surface glutamate receptors [33,34].

In the electron microscopic reconstruction, the PSD shows heterogeneous structures with dense domains and clear perforations, and can have a horseshoe-like structure with a notch [22]. It is not clear what such heterogeneity of electron density represents and how it affects the surface receptor distribution. It also remained to be elucidated how such subdomains are formed, especially because the PSD lacks membrane demarcation that can contain its components.

LLPS as a mechanism for the formation of liquid-like PSD protein condensate

LLPS is a phenomenon where liquid is spontaneously separated into two liquid phases, such as water and oil. It has been known in the field of soft matter physics but only recently has its significance in biological systems become widely acknowledged [35–38]. Early studies revealed that biological macromolecules, including nucleic acid and proteins, form condensate within the

cytosol [36,39]. Unlike an aggregate, the components in the condensate maintain their dynamics and native conformation. The components are freely mixing inside of the condensate and exchanging with other components from the surrounding environment.

The first observation which supported the idea that the PSD is formed by LLPS came from an experiment demonstrating LLPS of a protein solution containing two purified major PSD proteins, SynGAP and PSD-95 [40]. When these two proteins were mixed together, they spontaneously concentrated and formed droplets that are visible under light microscope. These droplets fuse together to form a larger droplet, indicating that they retain a property as a liquid. Consistent with the observation in the PSD, when fluorescently tagged SynGAP or PSD-95 in single condensates are photobleached, the fluorescence gradually recovers over time.

Subsequently, it was demonstrated that major PSD proteins, including GKAP, Shank, Homer, GluN2B [41], and Stargazin [42], also undergo phase separation through their multiplexed interactions and intrinsically disordered regions. Shank forms homooligomers through its SAM domain and Homer forms homotetramers through its coiled-coil domain [43]. When the multimer formation is prevented by introducing a mutation or competing with a monomeric Homer, the formation of LLPS is prevented [41]. At the same time, those mutants do not distribute at the synapse [43]. Thus, the interaction between Shank and Homer makes the macromolecular complex consistent with LLPS. On the other hand, the cytoplasmic regions of GluN2B and Stargazin do not have rigid conformations and are considered to be intrinsically disordered [24,42]. These regions are required for macromolecular complexes through LLPS.

CaMKII undergoes LLPS Ca^{2+} -dependently and forms phase-in-phase

Given that PSD proteins undergo LLPS, what confers the plasticity mechanism which persistently reorganizes the PSD in response to a neuronal stimulation? Indeed, CaMKII is a highly abundant multifunctional protein kinase essential for synaptic plasticity [44]. The glutamate released from the presynaptic side opens NMDAR and triggers Ca^{2+} influx to the postsynaptic side. Ca^{2+} /calmodulin binding to the regulatory domain of CaMKII activates the kinase activity. Once activated, CaMKII undergoes autophosphorylation at threonine (T) 286, which disables the autoinhibitory mechanism and makes the kinase constitutively active independent of Ca^{2+} [45]. Through this mechanism, it has been postulated that CaMKII is a memory molecule. Indeed, inhibition of CaMKII by inhibitors or genetical approaches impairs synaptic plasticity, learning and memory [46–48].

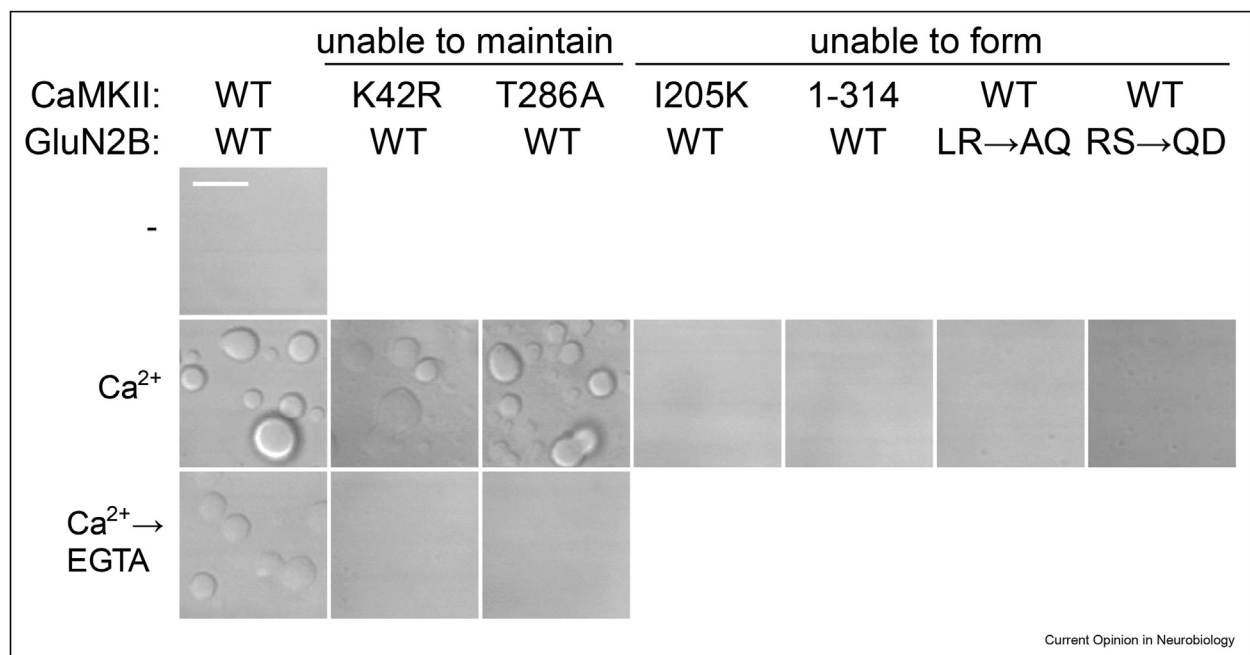
However, several mysteries remain for CaMKII. First, it is not known why CaMKII is so abundant in the PSD fraction. Indeed, CaMKII comprises 10–20% of PSD proteins and 2% of brain tissue [44,49]. In addition, it has a characteristic dodecameric structure not seen in other kinases [50]. Finally, CaMKII not only phosphorylates its substrates but also forms a stable complex through a binding pocket called the ‘T-site’ with some of the substrates, including the carboxyl tail of the NMDAR subunit GluN2B [51]. Hosokawa, Liu, *et al.* noticed that these features are ideal for undergoing LLPS.

To this end, purified CaMKII and the carboxyl tail of GluN2B (GluN2Bc) were tested for their ability to undergo LLPS *in vitro* (Figure 2). CaMKII or GluN2Bc alone or a mixture of these two proteins but without Ca^{2+} , did not condensate. However, when the mixture is stimulated by Ca^{2+} /calmodulin, they underwent LLPS (Figure 2). This requires specific interaction of the CaMKII T-site with GluN2Bc, as well as the multimeric structure of CaMKII (Figure 2). The formation of condensate is independent of kinase activity. However, autophosphorylation at CaMKII T286 is required to maintain the protein condensate after chelation of Ca^{2+} with EGTA. This may serve as a mechanism to

translocate CaMKII to the PSD near the source of Ca^{2+} for immediate activation. In addition to this, the T-site of CaMKII can interact with other proteins besides GluN2B such as Tiam1, densin-180, connexin-36, Rem2, and eag potassium channel [52–56]. Therefore, this binding may be a general mechanism to crosslink various postsynaptic proteins at the PSD. In addition, interaction between GluN2B and Tiam1 can maintain CaMKII in active conformation in the absence of Ca^{2+} /calmodulin though not to a full extent as in the presence of Ca^{2+} /calmodulin. Such basal activity in turn can maintain the phosphorylation of GluN2B and Tiam1, or potentially other synaptic proteins, thereby forming a reciprocally activating kinase–effector complex (RAKEC) [52].

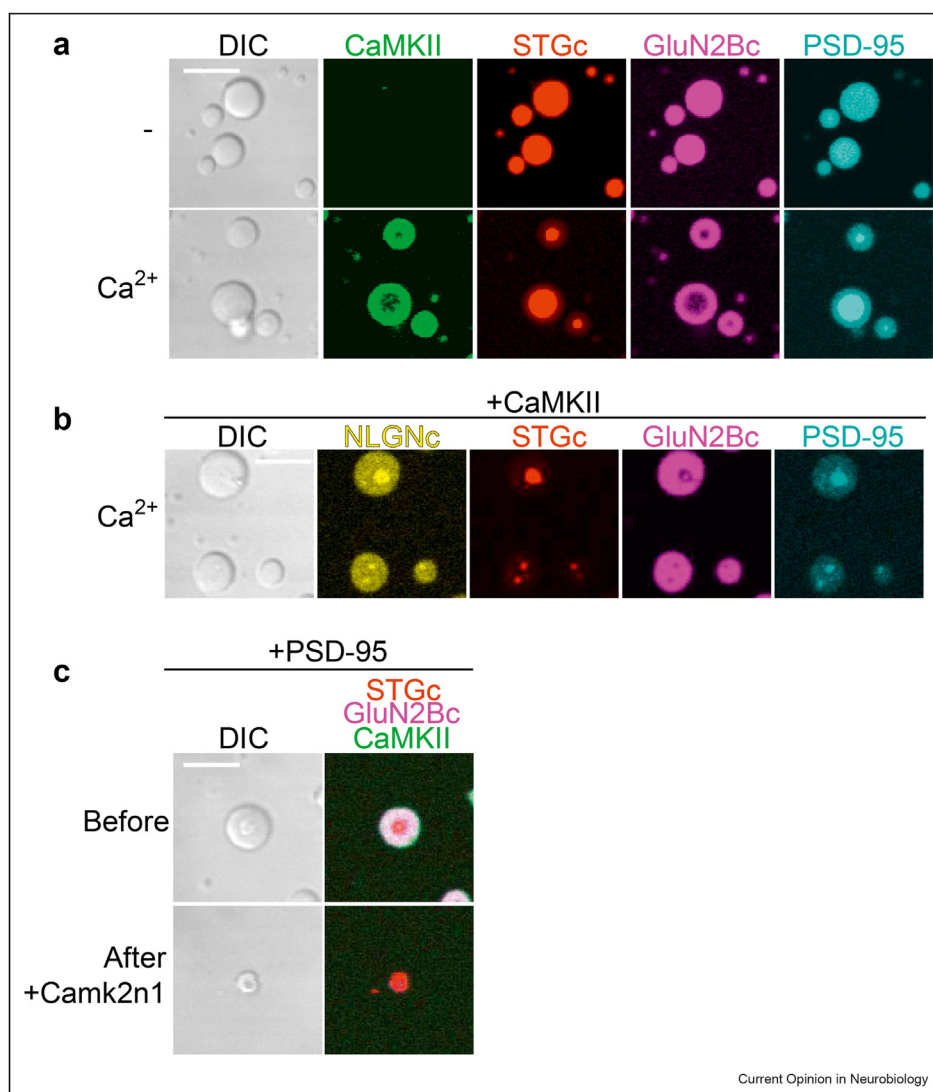
They further added AMPAR and scaffolding protein PSD-95 (Figure 3A). As a proxy for AMPAR, the carboxyl tail of Stargazin (STGc), an auxiliary subunit of AMPAR, was used because it is the major determinant of AMPAR distribution [57]. PSD-95 interacts with the PDZ-binding motif, which exists in both STGc and GluN2Bc, via its PDZ domain [57,58]. In the absence of Ca^{2+} , STGc, GluN2Bc, and PSD-95 formed a homogeneous condensate, whereas CaMKII remained in diluted phase. Interestingly, upon addition of Ca^{2+} , CaMKII

Figure 2



The formation and the maintenance of LLPS condensates. DIC images of the mixture of purified CaMKII and GluN2B by confocal fluorescent microscope. Wild-type (WT) CaMKII and WT GluN2B carboxyl tail form LLPS condensates by Ca^{2+} /calmodulin stimulation. Condensates were maintained even after the chelation of Ca^{2+} with EGTA. Kinase-null K42R and autophosphorylation deficient T286A were unable to maintain condensates, suggesting that the maintenance after the chelation of Ca^{2+} requires autophosphorylation. On the other hand, CaMKII T-site mutant I205K, monomeric 1–314 mutant and GluN2B interaction mutants LR→AQ, RS→QD unable to interact with CaMKII T-site failed to form condensate, suggesting that the formation requires multiplexed interaction between CaMKII T-site and GluN2B. Scale bar, 5 μm . LLPS, liquid–liquid phase separation.

Figure 3



The formation and disruption of phase-in-phase. (a) The activation of CaMKII resulted in the segregation of CaMKII-GluN2B LLPS condensate as surrounding phase from Stargazin-PSD-95 LLPS condensate as phase-in-phase structure. (b) Neurologin-1 participated in the phase-in-phase with Stargazin. (c) Injection of Camk2n1 protein, a source of CN21 sequence, to LLPS condensates forming phase-in-phase structure (Before) resulted in disruption of CaMKII-GluN2B surrounding phase whereas Stargazin-PSD-95 condensates remained (After). Scale bar, 5 μ m. LLPS, liquid-liquid phase separation.

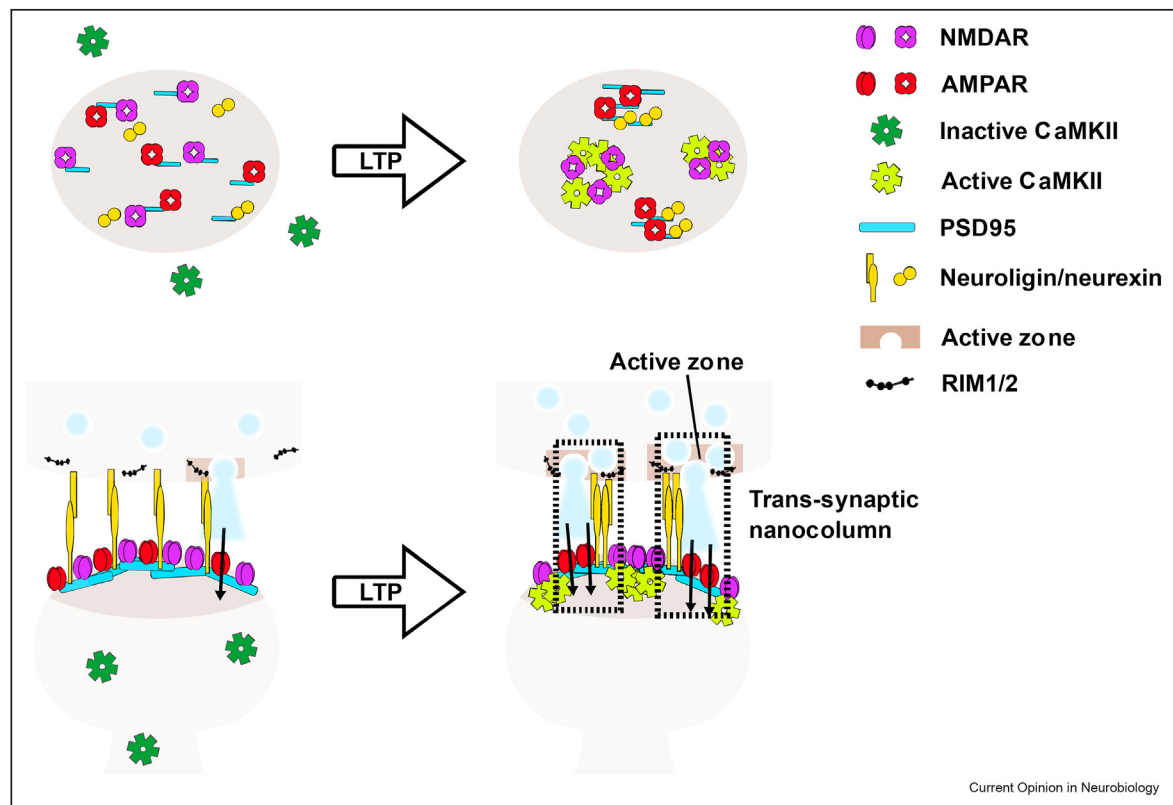
was incorporated into the condensed phase. At the same time, this caused a segregation of STGc from GluN2Bc. GluN2Bc and CaMKII distributed in the outer side of the condensate, whereas STGc and PSD-95 formed a separate phase inside of the condensate or a phase-in-phase structure. This indicates that activated CaMKII can segregate two types of glutamate receptors into two different phases by selectively binding only to NMDAR.

Intriguingly, CaMKII also undergoes LLPS when it is mixed with Shank3 [59]. In this case, however, the binding does not require activation by Ca^{2+} /calmodulin.

Therefore, depending on the activation status, CaMKII can undergo LLPS with different proteins. In its inactive state, it undergoes LLPS with Shank3 and when it is activated by Ca^{2+} /calmodulin it undergoes LLPS with GluN2B [59].

To confirm that the selective interaction of GluN2Bc with the T-site of CaMKII serves as the mechanism of segregation of AMPAR and NMDAR, we applied Camk2n1 which also interacts with the T-site of CaMKII and competes with GluN2B (Figure 3C). Infusion of Camk2n1 to protein condensates resulted in

Figure 4



A working model of the segregation of PSD proteins. Ca^{2+} influx during LTP induction triggers CaMKII-mediated LLPS and the segregation of AMPAR. This leads to the formation of trans-synaptic nanocolumn that ensures an efficient synaptic transmission. PSD, postsynaptic density; AMPAR, AMPA-type glutamate receptors

collapse of the surrounding CaMKII/GluN2Bc phase, while the PSD-95/STGc phase-in-phase structure remained. This indicates that LLPS of CaMKII and GluN2B is mediated by the CaMKII T-site. Consistently, GluN2B mutants with impaired T-site binding (LR→AQ and RS→QD mutants) failed to undergo LLPS (Figure 2).

Hosokawa, Liu, et al. then used CN21, a partial fragment of Camk2n1, to test if the segregation of AMPAR and NMDAR nanodomains is dependent on the CaMKII T-site or not. They incubated neurons with a cell-permeable form of CN21 (tat-CN21) and visualized the AMPAR and NMDAR by dual-color dSTORM imaging. As a result, we found that the segregation between these two classes of glutamate receptor was disrupted (Figure 1B), as shown in the overlap rate of nanodomains; AMPAR nanodomains overlapped with NMDAR nanodomains more in the tat-CN21 group (Figure 1C). This suggests that the interaction between the CaMKII T-site and GluN2Bc is critical for the segregation between NMDAR and AMPAR nanodomains. This is the first proposed mechanism of how

LLPS can regulate the formation of synaptic surface receptor nanodomains and how Ca^{2+} can modulate it.

Nanoscale organization between presynaptic and postsynaptic structures

Recent evidence indicates that pre- and post-synaptic nanodomains are functionally coordinated [8–10]. Tang, Chen, et al. proposed a ‘trans-synaptic nanocolumn’, a vertical alignment of presynaptic active zone protein and postsynaptic glutamate receptor [8,60]. Importantly, this alignment is altered during synaptic plasticity. When chemical LTP was induced by glycine stimulation, PSD-95 nanodomain was enriched beneath the active zone marker, Rab3-interaction molecule (RIM) 1/2 nanodomain. On the other hand, when chemical long-term depression (LTD) was induced by a prolonged activation of NMDAR [60], PSD-95 nanodomain was reduced. Interestingly, whereas RIM1/2 nanodomains stay constant in number after LTD induction, a retrograde realignment from postsynaptic to presynaptic counterpart was observed during the recovery period after LTD induction and the size of RIM1/2 nanodomains that align with PSD-95

nanodomains was increased [8]. Haas et al. [61] observed a colocalization of neuroligin 1 (NLGN1) clusters and AMPAR nanodomain at postsynaptic membrane and an alignment of NLGN1 cluster with presynaptic RIM1/2 nanodomain. Furthermore, expression of a carboxyl-terminally truncated form of NLGN1, which cannot interact with PSD-95, disrupted the colocalization with AMPAR nanodomain, as well as an alignment with RIM1/2 nanodomain. This resulted in a decrease in spontaneous EPSC amplitude [61]. These studies provide evidence for the existence of trans-synaptic nanocolumns which ensures efficient synaptic transmission. Because AMPAR is not saturated with glutamate at the synaptic cleft, the change in alignment of presynaptic transmitter release machinery with the postsynaptic AMPA receptor changes the efficacy of synaptic transmission [62–65].

A due question is whether LLPS formed by CaMKII is involved in the regulation of pre- and postsynaptic alignment (Figure 3B). Hosokawa, Liu, et al. decided to focus on NLGN1 because it interacts with presynaptic neuroligin, which further interacts with active zone machineries [66]. It was previously found that postsynaptic overexpression of NLGN1 increases the transmitter release probability via neuroligin [66]. Because NLGN1 also interacts with PSD-95 through its intracellular carboxyl tail, the behavior of NLGN1 was also tested in an LLPS experiment. Before the stimulation with Ca^{2+} , NLGN1 carboxyl tail (NLGN1c), STGc, GluN2Bc, and PSD-95 formed a homogeneous condensate. However, by the addition of Ca^{2+} and incorporation of CaMKII into the condensate, NLGN1c segregates together with AMPAR and PSD-95 from GluN2Bc. This result indicates that the activation of CaMKII segregates NLGN1 and AMPAR into the same nanodomain from CaMKII and GluN2B. The postsynaptic activation of CaMKII can support the formation of postsynaptic nanodomain and at the same time align the AMPAR nanodomain beneath the transmitter release site via a mechanism involving NLGN. This may serve as a novel mechanism of synaptic plasticity (Figure 4).

Conclusion

Recent progress in the understanding of LLPS can explain not only how the PSD forms but also how nanoscale structures of the PSD are regulated. The protein condensate formed by LLPS exhibits liquid-like properties such as both reactivity and plasticity while having stability, which makes it ideal for the molecular mechanism of synaptic plasticity and memory formation. Since protein molecules in the condensate show turnover between inside and outside, the condensate can overcome the lifetime of each protein molecule. This explains why we can maintain memory for our whole life while constituent molecules are turning over. Furthermore, LLPS can form protein complexes having

segregated subcompartments of proteins as phase-in-phase structures. This is likely to be the mechanism of activity-induced regulation of synaptic nanodomains. Emerging evidence suggests that the behavior of pre-synaptic active zone proteins and their function are also regulated by LLPS [67,68]. We expect LLPS will not only solve the long-standing mysteries of synaptic plasticity but also multiple others in the field of neuroscience.

Author contributions

P. L., T.H., and Y. H. jointly wrote the manuscript.

Conflict of interest statement

Y.H. is partly supported by Fujitsu Laboratories and Dwango.

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