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Presynaptic bouton compartmentalization and postsynaptic density-mediated glutamate receptor clustering via phase separation

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ABSTRACT

Neuronal synapses encompass three compartments: presynaptic axon terminal, synaptic cleft, and postsynaptic dendrite. Each compartment contains densely packed molecular machineries that are involved in synaptic transmission. In recent years, emerging evidence indicates that the assembly of these membraneless substructures or assemblies that are not enclosed by membranes are driven by liquid-liquid phase separation. We review here recent studies that suggest the phase separation-mediated organization of these synaptic compartments. We discuss how synaptic function may be linked to its organization as biomolecular condensates. We conclude with a discussion of areas of future interest in the field for better understanding of the structural architecture of neuronal synapses and its contribution to synaptic functions.

1. Introduction

Synapse is the communication hub between two neurons, allowing them to transmit information in the form of action potentials. The sending port, or the presynaptic bouton, is filled with synaptic vesicles (SVs) that contain neurotransmitters. Upon external stimuli, action potentials arrived at presynaptic bouton open the voltage gated calcium channel (VGCC), which triggers conformational change of the Ca^{2+} sensor, synaptotagmin1, and subsequently induction of SNARE-mediated fusion of SVs with the presynaptic membrane to release their contents. Neurotransmitters then diffuse across the synaptic cleft, binding to and activating postsynaptic receptors. Depending on the type of neurotransmitters and the membrane receptors they bind to, the effect on the receiving neuron can be either excitatory or inhibitory. In excitatory synapses, release of neurotransmitters triggers an influx of positively charged ions, leading to depolarization of the postsynaptic membrane and then the firing of action potential that is further travelled down the axon to pass information onto the next neuron. In inhibitory synapses, the release of neurotransmitters induces an influx of negatively charged ions that leads to hyperpolarization of the postsynaptic membrane, raising the threshold to fire an action potential. Synapses are extremely plastic, and the efficiency of how two neurons communicate with each other can be tuned up or down (as in long term potentiation (LTP) or depression (LTD)) for adaption to different brain activities. Human brain comprises billions of neurons, each of which can make connections to thousands of others. It is this tremendous pattern of synaptic connections and the extreme plasticity determine the exceptionally complex neuronal circuits and functions.

Unlike other membrane enclosed or membraneless organelles, synapses take a unique semi-membrane bound type of compartmentalization. Beneath the presynaptic membrane, a layer of electron dense material, known as active zone (AZ), could be observed by electron microscope (EM) and different types of synapses have different shapes of AZ (Akert et al., 1971; Harlow et al., 2001; Kittel et al., 2006; Matthews and Fuchs, 2010; Sudhof, 2012b; Zhai and Bellen, 2004). In mammalian hippocampal synapses, AZ appears as punctuated dense projections with intercalated vesicles ready to fuse with the plasma membrane for neurotransmitter release (Akert et al., 1971; Limbach et al., 2011; Pfenninger et al., 1972). The AZ area is spatially separated from the rest of the presynaptic bouton where reserve pools of SVs are clustered, yet it is accessible for vesicle exchange due to the absence of a delineating membrane (Fig. 1). Beneath the postsynaptic membrane lies a densely packed, protein rich, mega complex structure of about hundred nanometers in width and ~30–50 nm in thickness, known as the postsynaptic density (PSD) (Fig. 1) (Blomberg et al., 1977; Carlin et al., 1980; Chen

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Abbreviations

SV	Synaptic vesicle
VGCC	voltage gated calcium channel
LTP	Long term potentiation
LTD	Long term depression
AZ	Active zone
EM	Electron microscope
PSD	Postsynaptic density
ePSD	Excitatory PSD
iPSD	Inhibitory PSD
LLPS	Liquid-liquid phase separation
IDR	Intrinsically disordered region
PRM	Proline rich motif
SLB	Supported lipid bilayer
GUV	Giant unilamellar vesicle
SUV	Small unilamellar vesicle
CLEM	Correlative light-electron microscopy

et al., 2008; Cohen et al., 1977; Gray, 1959; Harris and Weinberg, 2012; Palay, 1956). Genetic and cellular studies in the past decades have identified key proteins essential for AZ and PSD assembly and function. Biochemical and structural studies have provided further mechanistic insights into how protein interactions contribute to synaptic organization.

Studies in recent years provide substantial evidence showing that assembly of both pre- and post-synaptic compartments is driven by liquid-liquid phase separation (LLPS) (Chen et al., 2020; Feng et al., 2018, 2019b; Wu et al., 2020). Phase separation happens when a solution demixes under certain condition (e.g. above a threshold concentration of solutes), resulting in a dilute phase co-existing with a dense phase (Banani et al., 2017; Lyon et al., 2020; Shin and Brangwynne, 2017). The LLPS model is consistent with the in vivo observations that protein constituents within AZ and PSD are at higher local concentrations than the surrounding environment, sharp concentration gradients are stably maintained, and dynamic protein exchange occurs both within and between compartments. We are only now beginning to understand key organizational principles of these semi-membrane bound biomolecular condensates. Glutamate is the most prominent excitatory neurotransmitter in vertebrate nervous system, and here in this review we focus on this type of synapses. We highlight recent findings on how phase separation underlies the organization of synaptic machineries including presynaptic AZs, clustered SV pools, and excitatory PSDs (ePSDs) (Fig. 1). We also discuss how the LLPS model aligns with findings from earlier studies on the assembly mechanism of neuronal synapses. We further underscore key outstanding questions that remain to be answered for understanding the structural organization of glutamatergic synapses. As the prototypical biomolecular condensate, insights into the structure and function of synapses might help uncover the biophysical principles and specific properties that drive the formation of other membraneless or semi-membrane bound compartments in cells.

2. Structural organization of the presynaptic terminal

The presynaptic terminal is the information sending port of a synapse. Action potentials travel along the axon reaching to the terminal region where AZ proteins and primed SVs localize. SVs are packed with neurotransmitters, the information carriers that bind to and activate postsynaptic receptors on the receiving neuron. The AZ refers to a specialized area of the presynaptic membrane where SVs dock and fuse to release contents (Fig. 2A) (Jahn and Fasshauer, 2012; Sudhof, 2012b). Action potentials open VGCCs, which then triggers Ca^{2+}-dependent vesicle exocytosis and the release of neurotransmitters into the synaptic cleft. Therefore, one of the major functions of the AZ is to link VGCCs to the docked and primed SVs for fast and efficient release (Brunger et al., 2018; Sudhof, 2012a, 2013). Functionally, the AZ also ensures precise alignment of vesicle release sites with clustered postsynaptic AMPA receptors, potentially via synaptic adhesion molecules, for information transfer between two cells, although the underlying mechanism is still poorly understood (Biederer et al., 2017; Sudhof, 2018). The AZ appears as a thin layer of electron dense material attached to the membrane and is positioned opposite to the PSD. EM studies of central synapses revealed that AZ comprises spatially separated dense projections with docked vesicles that are ready to fuse with the axon terminal plasma membranes (Imig et al., 2014; Siksou et al., 2007). The biochemical composition of the AZ has also been studied (Wilhelm et al., 2014). The core of AZs is composed of a detergent insoluble protein matrix that is difficult to purify, but which comprises five evolutionarily conserved proteins: RIM, RIM-BP, ELKS, liprin-α and Munc13. These proteins, via pairwise domain interactions, assemble into a large protein complex that tethers and docks SVs, locates and clusters VGCCs, and aligns presynaptic terminal to PSDs (Emperador-Melero and Kaeser, 2020; Sudhof, 2012b).

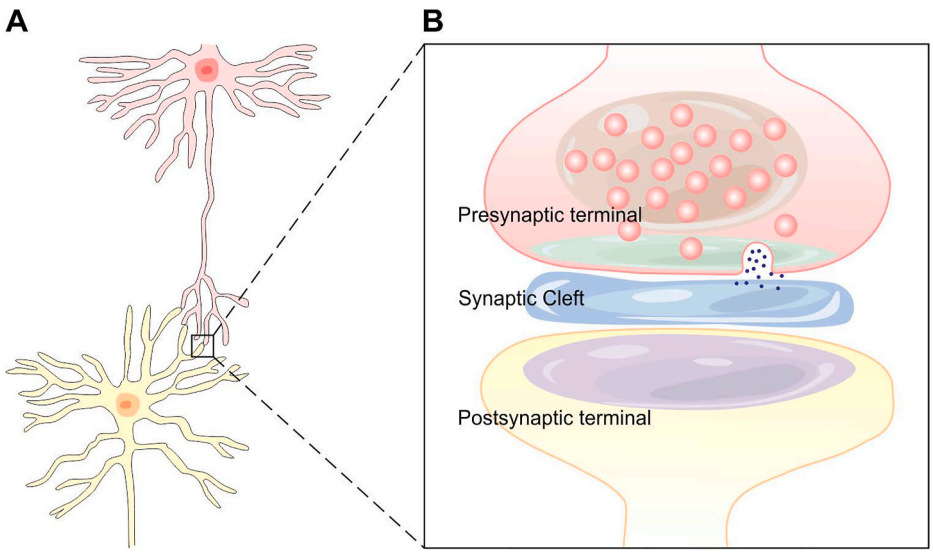

Fig. 1. Schematic diagram of phase separation-mediated synaptic compartmentalization.
A. Schematic diagram of a chemical synapse. A presynaptic neuron (pink) projects to a postsynaptic neuron (yellow) and form synapses between them. B. Representative phase-separated condensates at a glutamatergic synapse. Phase separation was reported to be the plausible mechanism for pre- and post-synaptic terminal organization. It is proposed that the formation of synaptic cleft might also employ this principle.

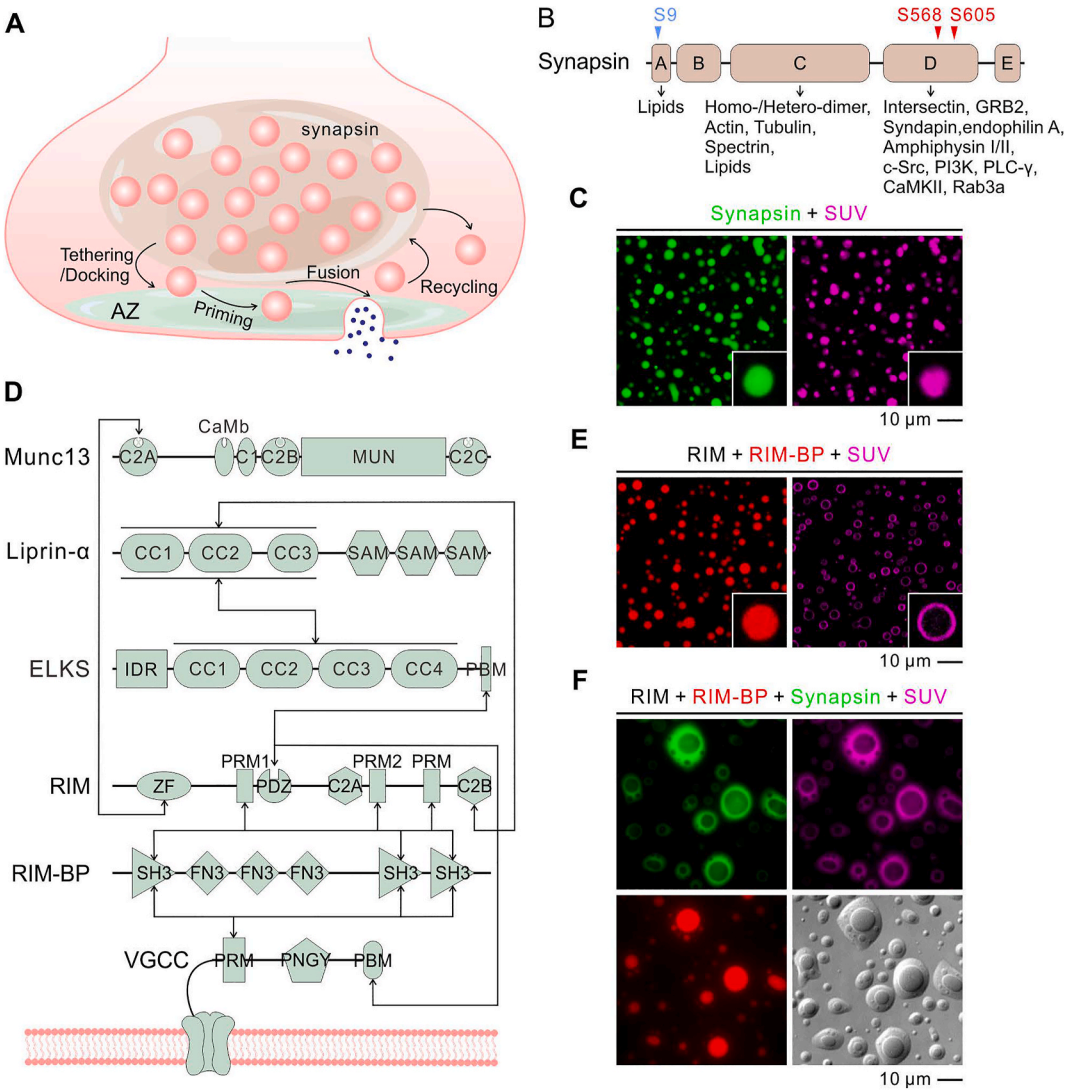

Fig. 2. Phase separation at a presynaptic terminal.
A. Schematic drawing of phase separation-mediated presynaptic terminal organization and SV cycling. Synapsin I has been shown to undergo phase separation and to cluster reserve pool SVs. CaMKIIα-mediated phosphorylation dissolves synapsin condensates and releases more SVs accessible to the presynaptic plasma membrane. Major scaffold proteins in the AZ were shown to undergo phase separation and tether SVs to the plasma membrane. When mixed together, synapsin and RIM–RIM-BP condensates were immiscible, with the synapsin condensates encapsulating the RIM–RIM-BP droplets. SVs can travel from the reserve pool to the docked pool for multiple cycles of release events. It remains unknown whether and how phase separation contributes to the process of SV priming, fusion, and recycling.B. Domain architecture of synapsin. Synapsin contains A, B, C, D, and E, five domains. Its known binding partners are listed below each domain. Above the domain marks the better studied phosphorylation sites. Red colour marks the sites phosphorylated by CaMKIIα, and the blue colour marks the site phosphorylated by PKA. Numbers are in accordance with human synapsin Ia (Uniprot #P17600). C. Representative images showing the co-enrichment of synapsin condensates with SUVs. The insert shows an enlarged view of a droplet.D. Domain architecture and interaction network of the five major active zone scaffold proteins and the α1 subunit of voltage gated Ca^{2+} channel (VGCC). Black arrows indicate interactions between indicated domains. E. Representative images of RIM–RIM-BP phase separation droplets and the coating of SUV on their surface. The insert shows an enlarged view of a RIM–RIM-BP droplet coated by SUV.F. Representative images of immiscible synapsin-SUV coacervates and SUV coated RIM–RIM-BP droplets.

2.1. SV clustering driven by protein phase separation

At the presynapse, vesicles can freely translocate between different pools, each having distinct functions: the reserve pool, the recycling pool, and the readily releasable pool (Alabi and Tsien, 2012; Milovanovic and De Camilli, 2017) (Fig. 2A). Vesicles are densely packed in the presynaptic termini without clear delineation from the surrounding cytoplasm. Synapsin is an extremely abundant protein in the presynaptic bouton (Fig. 2B), and is a major constituent of the scaffolding platform that clusters reserve pool SVs (Cesca et al., 2010; Wilhelm et al., 2014), as its disturbance by genetic knockout or antibody injection would specifically disperse SVs in the reserve pool (Pieribone et al., 1995; Rosahl et al., 1995). Synapsin contains a C-terminal intrinsically disordered region (IDR) and multiple SH3 domain binding proline rich motifs (PRMs) (Cesca et al., 2010; Pechstein et al., 2020), a sequence feature characteristic of many phase separation-prone proteins (Banani et al., 2017; Gomes and Shorter, 2019). In vitro experiments showed that EGFP-tagged synapsin protein can undergo LLPS in the presence of low concentration of crowding reagent (3% PEG8000) with or without its binding partners such as intersectin and GRB2, both of which are components of the protein network that facilitates SV clustering (Milovanovic et al., 2018). The resulting condensates recruit and concentrate vesicles through interaction with negatively charged phospholipids on the vesicle surface (Fig. 2C). Both proteins and liposomes are mobile in the dense phase. EM analysis further demonstrated that vesicles are clustered by synapsin both in vitro and in synapses from excitatory and

inhibitory nerve terminals isolated from adult mice (Milovanovic et al., 2018). Microinjection of antibodies or fusion proteins that specifically target the IDR in synapsin led to dispersion of SV clusters in living neurons (Pechstein et al., 2020). Ectopically expressed synapsin and synaptophysin cooperatively resulted in small vesicle clusters in COS7 cells (Park et al., 2021). These cell-based studies further support the role of phase separation in synapsin-mediated SV clustering.

Synapsin is a substrate of CaMKII (Fig. 2B) (Benfenati et al., 1992; Nichols et al., 1990), and is phosphorylated upon presynaptic stimulation that triggers the opening of VGCCs and the subsequent influx of Ca^{2+}. Phosphorylation on synapsin by CaMKII results in its dissociation from SVs and dispersion within the nerve-terminal cytosol, and consequently more vesicles become available for exocytosis (Nichols et al., 1990). In vitro the addition of CaMKII, together with Ca^{2+}/CaM and Mg^{2+}-ATP, leads to rapid disassembly of synapsin phase condensates and dispersion of associated vesicles. Importantly, this phosphorylation-mediated dissolution is CaMKII specific as addition of an unrelated kinase, PKC, did not affect synapsin−liposome co-condensation (Milovanovic et al., 2018). However, whether and how another protein kinase, PKA, which is known to phosphorylate synapsin at other sites and modulate synapsin−SV association in situ (Fig. 2B), might regulate the phase separation of synapsin−SV remains to be addressed in the future (Hosaka et al., 1999).

2.2. Phase separation drives active zone assembly

SVs enriched by synapsin condensates are in close contact with the AZ, which is plausibly another biomolecular condensate attached to the membrane (Wu et al., 2019). The AZ contains five major scaffold proteins, including RIM, RIM-BP, ELKS, liprin-α and Munc13 (Fig. 2D). These proteins are highly conserved through evolution. For vertebrates, two additional scaffolds, Bassoon and Piccolo are also specifically positioned at the AZ and often used as AZ markers (Gundelfinger et al., 2015; Sudhof, 2012b). Using 3D-STORM combined with immunolabelling, RIM proteins were found to enrich within subregions of the presynaptic AZ, correlating with the fusion sites for SV exocytosis and directly opposite to the postsynaptic receptors and condensed scaffolds (Tang et al., 2016). VGCCs are also clustered within these nanodomains to facilitate efficient and precise neurotransmitter release at fusion sites (Dolphin and Lee, 2020; Eggermann et al., 2011; Miki et al., 2017; Nakamura et al., 2015).

In vitro purified RIM and RIM-BP proteins assemble into liquid-like condensates when mixed at near physiological concentrations (Wu et al., 2019). These phase droplets show fast exchange with materials in the surrounding solution, fuse and coalescence upon contact. RIM protein contains above average prolines in its amino acid sequence composition, and its multiple PRMs bind to the SH3 domains in RIM-BP (Fig. 2D). RIM−RIM-BP phase separation is therefore driven by multivalent, specific SH3-PRM interactions, together with weak interactions via IDRs. Genetic experiments revealed that RIM and RIM-BP proteins are essential for the proper localization and clustering of VGCCs to the AZ and for tethering SVs to the fusion site near VGCCs (Acuna et al., 2015, 2016; Kaeser et al., 2011). In vitro, RIM−RIM-BP condensates recruit and cluster the cytoplasmic tails of VGCCs (VGCC-CTs) via direct PDZ-PBM and PRM-SH3 interactions, and VGCC-CTs in return promote phase separation within the system (Wu et al., 2019). It should be noted that VGCC-CT itself is not required for RIM−RIM-BP condensation, in line with a recent finding that genetic removal of neuronal specific VGCCs does not influence the formation of AZ nano-assembly (Held et al., 2020). On the supported lipid bilayer (SLB), membrane attached VGCC-CTs are clustered into stable microdomains by RIM and RIM-BP, showing much higher density and slower diffusion rates than proteins in the dilute phase. The density of VGCC-CTs on the membrane is estimated to be comparable with that measured by EM-based methods on neuronal synapses (Miki et al., 2017; Nakamura et al., 2015). Similarly, purified RIM and ELKS proteins can also undergo LLPS under near physiological conditions (Wu et al., 2021).

Using this reconstituted AZ system, it has been shown that RIM−RIM-BP condensates specifically tether, but not coacervate with, SVs (Wu et al., 2021) (Fig. 2E). Proteins can still freely enter or escape from the SV-coated condensed phase. The observation of SVs coating on the surface of RIM−RIM-BP condensates is in vast contrast to the co-condensation of synapsin and SVs (Fig. 2C vs. Fig. 2E). Remarkably, it was shown that synapsin and RIM−RIM-BP condensates were immiscible when mixed under physiological buffer conditions (Fig. 2F). The synapsin condensates encapsulate the RIM−RIM-BP condensates, analogous to their co-existence in presynaptic boutons. A synapsin fragment containing the dimerization domain C and the intrinsically disordered domain D (termed as “synapsin CD”) became fully miscible with the RIM−RIM-BP condensates, suggesting that other short, unstructured regions outside the CD part of synapsin are required for multiphase immiscibility. Furthermore, SVs are specifically enriched in the synapsin sub-compartment but do not enter the inner RIM−RIM-BP condensates. Both condensates are dynamic as demonstrated by FRAP analysis and competing peptide-induced dispersion experiments. Further reconstitution on giant unilamellar vesicles (GUVs) illustrated how RIM−RIM-BP droplets tether to the GUV surface via membrane attached VGCC-CTs or phosphatidylinositol phosphate on one side and coated by small unilamellar vesicles (SUVs) on the other side. Such SUV-coated phase droplets were further surrounded by synapsin−SUV condensates. This reconstitution system, constituted of only a minimal set of components, recapitulates the multiphase organization observed in presynaptic boutons (Wu et al., 2021). The study also provides an example showing distinct modes of interactions between membrane-demarcated organelles (e.g., SVs and plasma membranes) and membraneless condensates (e.g., synapsin condensates and RIM−RIM-BP condensates) in a tiny space of a presynaptic bouton. One might imagine that, in cells, numerous forms of interaction modes between membrane-based organelles and membraneless biological condensates may exist.

The tiny size and limited number of molecules within a synapse make it difficult to probe the phase separation theory in living neurons, but emerging evidence from super-resolution imaging, correlative light-electron microscopy (CLEM) and ectopic expression in heterologous cells supports that phase separation occurs in vivo (Carvalhais et al., 2021). A recent study directly tested the biological importance of phase separation at synapses in mice (Emperador-Melero et al., 2020). Liprin-α is one of the major scaffold proteins at the AZ, with α2 and α3 isoforms reside predominantly in the brain (Serra-Pages et al., 1998). Liprin-α3 is localized at the AZ while α2 translocates from a distal region to the AZ after removal of α3 (Wong et al., 2018). It was found that PKC-mediated phosphorylation at a single site on liprin-α3 triggers its phase separation in HEK293 cells (Emperador-Melero et al., 2020). The spherical morphology of droplets, the sharp contrast between the droplets and the surrounding under CLEM, their fusion upon contact and fast protein exchange with the surrounding cytosol are all indicative of LLPS. Co-expression of RIM and Munc13, two other important AZ proteins, showed co-localization with liprin-α3 condensates. In liprin-α2 and -α3 double knockout mice, synaptic enrichments of RIM and Munc13 were diminished, the number of docked SVs was reduced, and neurotransmitter release was impaired. PKC phosphorylation on liprin-α3 could upregulate the amount of RIM and Munc13 proteins recruited to the AZ and enhance the level of neurotransmitter release. Altogether these findings suggest that liprin-α3 undergoes phosphorylation-regulated phase separation in presynaptic terminals to modulate AZ structure and function (Emperador-Melero et al., 2020).

In addition to vertebrates, phase separation of liprin-α has also been indicated by another recent study in *C. elegans* (McDonald et al., 2020). The SYD-2 (mammalian liprin-α ortholog) and ELKS mutants that specifically disturbed LLPS of the mixture in vitro failed to recruit and properly assemble other AZ components such as UNC-10 (mammalian RIM ortholog) and UNC-13 (mammalian Munc13 ortholog) in vivo.

These defects could be rescued by introducing an IDR from the unrelated FUS protein into the syd-2 mutant, suggesting that phase separation of SYD-2 is essential for AZ assembly.

Although the molecular composition of the AZ is well studied, it is puzzling that the knockout of individual proteins or family of proteins does not significantly affect AZ assembly, but simultaneous removal of several genes can result in severe structural and functional defects of AZ (Acuna et al., 2016; Wang et al., 2016). This suggests that the protein interaction network, rather than individual proteins, are important for the organization of the AZ platform. The concept of phase separation might help to explain these in vivo observations (Chen et al., 2020; Wu et al., 2020). Specifically, it is likely that the removal of any of the major scaffold protein is not able to abolish the entire scaffolding network, as other pairs of interactions are maintained to drive phase separation and thus the AZ scaffold assembly. Phase separation also enables co-existence of multiple phases in a single spatial compartment even in the absence of a membrane barrier (Wu et al., 2021). Strikingly, different protein condensates demonstrate distinct modes of interaction with membrane-based organelles (Wu et al., 2020; Zhao and Zhang, 2020), a phenomenon hard to be explained by canonical interactions in a homogenous solution state.

Many important questions remain in the study of phase separation-driven structural organization within presynaptic terminals. Several of the key AZ scaffold proteins, including ELKS, Munc13-1 and Liprin-α,

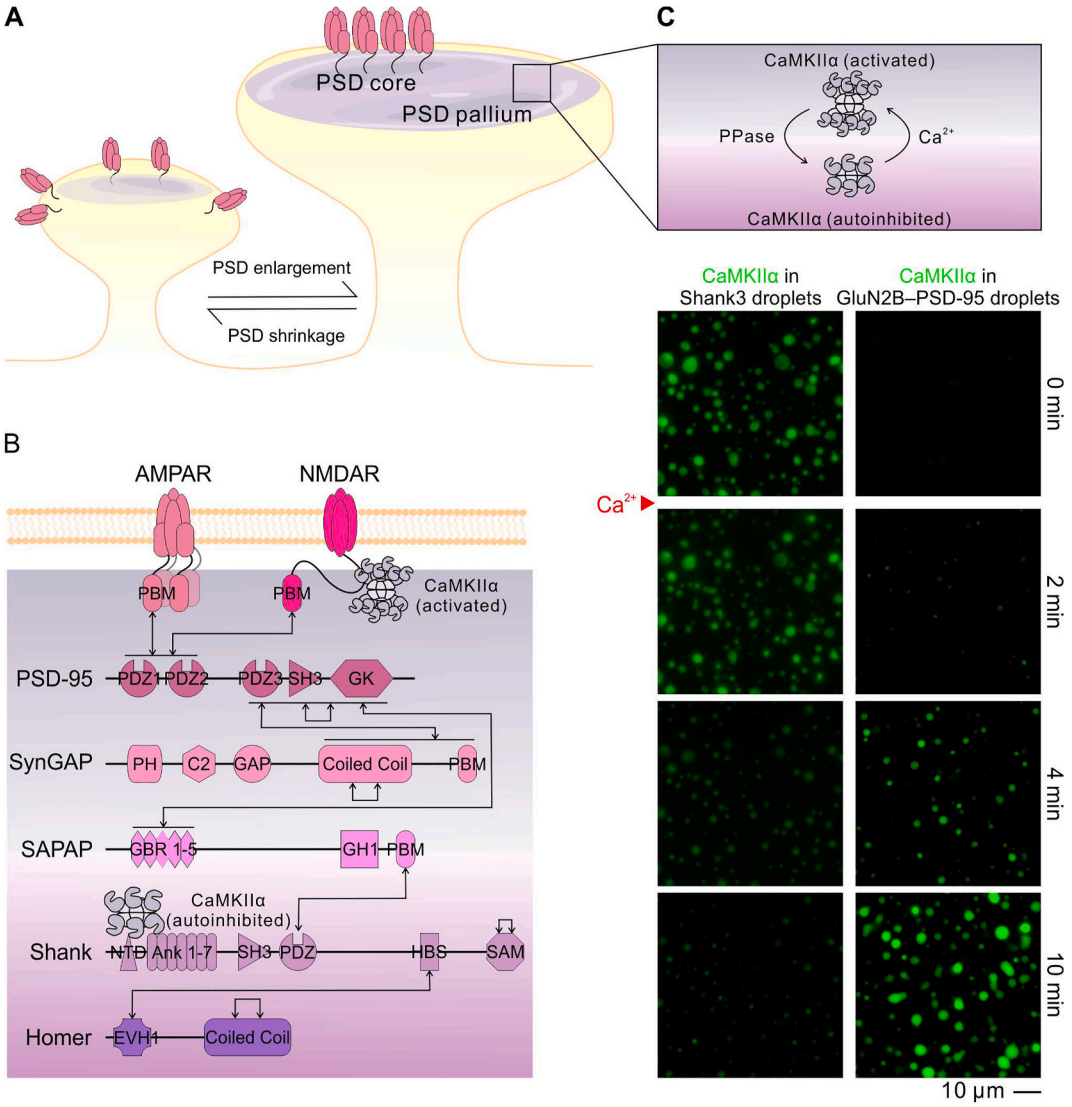

Fig. 3. Phase separation-mediated ePSD organization.

A. Schematic drawing of phase separation-mediated ePSD organization. ePSD exhibits layered organization with two layers known as the core and the pallium, which involves different scaffold proteins bridged by scaffold linkers. Via biochemical reconstitution, it was revealed that PSD assembly and organization are driven by phase separation. PSD condensates can cluster and enrich postsynaptic membrane receptors into the synaptic region. The ePSD condensates can be modulated by activity-dependent protein modifications. Increases in the amount of local protein synthesis or CaMKII-mediated phosphorylation of scaffold proteins can enhance the phase separation capacity within the system, leading to increase in the PSD area and enlargement of the spine head volume. On the other hand, upregulation of Homer 1a expression or ubiquitin-mediated protein turnover can decrease the phase separation capacity within the system, leading to PSD shrinkage. AMPA receptors traffic to the synaptic region via phase separation when the synapse is strengthened and disperse to the extrasynaptic region for endocytosis when the synapse is weakened.B. Domain architecture and interaction network of the major ePSD scaffold proteins. Black arrows indicate interactions between indicated domains. C. Upper panel, schematic model showing the shuttling of CaMKIIα between the core and the pallium layers depending on its conformational states/binding partners. Ca^{2+} activated CaMKIIα binds to the cytoplasmic tail of GluN2B and is enriched within the PSD core, whereas dephosphorylated, autoinhibited CaMKIIα binds to and phase separates with Shank3 to be retained in the PSD pallium. Lower panel, representative images showing Ca^{2+} injection triggered dispersion of CaMKIIα from Shank3 condensates but enrichment within GluN2B–PSD-95 droplets. Images were taken from (Cai et al., 2021) with permission.

were observed to assemble into liquid-like condensates in heterologous cells or living neurons (Emperador-Melero et al., 2020; Sakamoto et al., 2018; Sala et al., 2019). But whether these proteins, each biochemically purified to high purity and homogeneity, is sufficient to undergo phase separation on their own or multiple proteins need to be mixed together to trigger phase separation, remains largely unknown. Apart from these key AZ scaffold proteins, do other AZ proteins, such as Basson and Piccolo, and the SNARE fusion machinery undergo LLPS and how do they contribute to the phase separation-driven AZ assembly? In addition, evidence emerging from in vivo studies raises the possibility that the AZ might contain multiple independent phases (Emperador-Melero et al., 2020). Depletion of liprin-α2 and -α3 reduced the localization of RIM and Munc13 at the AZ, but not of RIM-BP, and the levels of P/Q-type of VGCCs are increased. Similarly, removal of RIM and RIM-BP or RIM and ELKS does not affect presynaptic recruitment of liprin-α. It is possible that RIM−RIM-BP−ELKS condensates might co-exist with another type of condensates that are comprised of RIM, Munc13 and liprin-α. How does RIM discriminate these compartments? And how these different subcompartments interact with each other? Using a minimal set of presynaptic components, we have observed coating of SVs on the surface of AZ condensates. In addition to RIM, RIM-BP and ELKS included in these reconstitution assays, how do other proteins, such as Munc13 (a priming factor for SVs), synaptotagmin (a Ca^{2+} sensor), small GTPases and so on, participate in the docking and priming processes of SVs?

3. Structural organization of the postsynaptic terminal

The postsynaptic terminal, the information receiving port of synapses, is another example of a semi-membrane bound compartment thought to be formed via the process of LLPS (Fig. 3A). Underneath the postsynaptic plasma membrane lies a prominent structure that appears electron dense under EM and is resistant to detergent wash during purification (Blomberg et al., 1977; Carlin et al., 1980; Chen et al., 2008; Cohen et al., 1977; Gray, 1959; Harris and Weinberg, 2012; Palay, 1956). This layer of material is termed as the PSD and concentrates thousands of proteins to receive, process and converge signals from the presynaptic terminal to the next neuron (Kennedy, 2000; Sheng and Hoogenraad, 2007; Sheng and Kim, 2002). Super resolution microscopy has revolutionized the characterization of ePSD protein complexes and uncovered the presence of nanodomains within ePSDs (Chen et al., 2008; Dani et al., 2010; Harris and Weinberg, 2012; Huganir and Nicoll, 2013; MacGillavry and Hoogenraad, 2015; Maglione and Sigrist, 2013; Nair et al., 2013; O'Rourke et al., 2012; Sigrist and Sabatini, 2012; Triller and Choquet, 2008). AMPARs show a diffusive localization with high mobility in the extrasynaptic membrane fraction, but much slower dynamics at the synaptic region. PSD95, a major organizer of the ePSD structure, assembles into nanoclusters with limited diffusion rates as demonstrated by single molecular tracking experiments. The other three prominent ePSD scaffold proteins, Shank, GKAP (aka SAPAP or DLGAP) and Homer, assemble into the same nanocluster with PSD-95 showing layered architectural patterning (Fig. 3B). The ePSD is spatially separated from the rest of the spine cytoplasm, yet it is accessible for dynamic exchange due to the absence of delimiting membranes. During LTP or LTD, the spine head volume is strongly correlated with the size of the PSD and the amount of protein constituents (Bosch et al., 2014; Kasai et al., 2003; Meyer et al., 2014). Earlier biochemical and structural studies demonstrated that the ePSD scaffold proteins contain modular domains involved in protein interactions (Feng and Zhang, 2009; Zhu et al., 2016). Many of these binding domains are in tandems and can specifically interact with a variety of target proteins, although some with moderate binding affinities. Homer and Shank together form a mesh-like matrix structure, and Homer tetramerization is essential for this assembly (Hayashi et al., 2009). In neurons, the tetramerization of Homer is required for the structural integrity of dendritic spines and the recruitment of Shank and Homer to synapses. This earlier study showed that ePSD scaffold proteins assemble into large molecular complexes.

3.1. Phase separation drives postsynaptic density assembly

The initial observations of PSD organization via biomolecular condensation came from a recent study on the interaction between PSD-95 and SynGAP (Zeng et al., 2016). SynGAP is a negative regulator of synaptic strengths through downregulating activities of small G proteins (Araki et al., 2015; Vazquez et al., 2004). It is highly abundant in ePSD, with a near stoichiometric ratio to PSD-95 (Cheng et al., 2006). In vitro purified PSD-95 and SynGAP proteins condensate into spherical droplets, and this process is mediated by a specific PDZ-PBM interaction and the trimeric coiled-coil domain on SynGAP. Initial work did not use full length proteins (SynGAP CC-PBM and PSD-95 PDZ3-SH3-GK fragments were used). Including the first two PDZ domains of PSD-95 further increased the valency in the system and thus promoted the phase separation of the complex. LLPS-blocking mutations, which specifically target the oligomerization interface but not the PSD-95 binding interface on SynGAP, reduced its synaptic localization at basal state in neurons. Upon chemical LTP induction, a larger fraction of the SynGAP mutants were dispersed from synapses, and the neurons had enlarged spines and were in a hyper-excitation status when compared to the wild type group. These results suggest that SynGAP−PSD-95 phase separation is essential for their synaptic localization and subsequently to regulate synaptic strengths under physiological conditions. The study also implies that the formation of the mega-ePSD assembly may be mediated by phase separation.

Following the initial study showing the importance of phase separation in ePSD assembly, we recently reconstituted the ePSD condensate biochemically, via multivalent interactions between the major PSD scaffold proteins- PSD-95, GKAP, Shank and Homer (Zeng et al., 2018). These reconstituted condensates recruited and clustered receptor proteins (as exemplified by the cytoplasmic tail of GluN2B), concentrated cortactin, an activator of the actin nucleating Arp2/3 complex, and accelerated actin polymerization. ePSD condensates partially excluded gephyrin, an abundant scaffold protein that functions specifically in inhibitory synapses. This exclusion potentially explains why excitatory and inhibitory synapses do not overlap with each other in neurons, although the underlying mechanistic details are awaiting to be addressed. Another example of how ePSD condensates exhibit activities that cannot be achieved with a dilute, homogenous solution state came from the observation that the phase separation capacity could be modulated by adjusting the molar ratio of Homer1a to Homer1c in the system. Homer1a is a monomeric splice isoform of the tetrameric Homer1c protein (Xiao et al., 2000), albeit both isoforms can bind to Shank with the same affinity. Overexpression of Homer1a is known to cause shrinkage in spine head volume in neurons. Recent work also indicated that the accumulation of Homer1a at ePSDs is correlated with the global downscaling of PSD sizes during sleep in mice (de Vivo et al., 2017; Diering et al., 2017). Addition of Homer1a dispersed reconstituted ePSD condensates in solution and on supported membranes (Zeng et al., 2018). It is striking that the influence on a single interaction node can have a global architectural role on the entire interaction network. To further recapitulate the membrane attachment of ePSD in synapses, reconstituted PSD condensates were tethered on SLBs via membrane-attached GluN2B tail (Zeng et al., 2018). On the two-dimensional membrane, PSD scaffold proteins underwent phase separation via spinodal decomposition at considerably lower concentration compared to the 3D solution.

AMPA receptors are targeted to, and clustered within, the synaptic site via interactions with a group of scaffold proteins known as the DLG subfamily of MAGUKs, with PSD-95 as the prototype member (Chen et al., 2015; Elias et al., 2006; Elias and Nicoll, 2007; Feng and Zhang, 2009; Levy et al., 2015). Interestingly, PSD-95 does not directly bind to the cytoplasmic tail of AMPARs, but via interaction with AMPAR auxiliary subunits termed TARPs (Greger et al., 2017; Jackson and

Nicoll, 2011). Members of the TARP family proteins share a similar domain organization, with four transmembrane segments for interaction with AMPARs and a long unstructured cytoplasmic tail for interaction with MAGUKs (Bats et al., 2007; Chen et al., 2000; Dakoji et al., 2003; Schnell et al., 2002). Earlier genetic and cellular studies have shown that TARPs/MAGUKs interaction with AMPARs is essential for many functional aspects of the receptors (Greger et al., 2017; Jackson and Nicoll, 2011), but our understanding of the molecular determinants contributing to the specific and direct interactions between TARPs and MAGUKs is extremely limited. A recent study revealed that TARP family members can undergo LLPS with PSD-95 in vitro, mediated by a cluster of Arg residues upstream its C-terminal PDZ binding motif, in a protein and salt concentration-dependent manner (Zeng et al., 2019). The cytoplasmic tail of TARP proteins was further enriched into reconstituted ePSD condensates via binding to PSD-95. On the reconstituted membranes, TARP-CT formed sub-micron sized clusters with co-enrichment of other PSD scaffolds; neutralization of the Arg-rich motif diminished the TARP−PSD LLPS capabilities on lipid bilayers. In living neurons, transfection with TARP−PSD-95 LLPS deficient mutants impaired AMPAR EPSCs and produced no potentiation to synaptic strengths. Altogether, these findings demonstrate that the multivalent interaction between PSD-95 and TARP and the subsequent AMPAR clustering by PSD condensates are essential for the targeting and clustering of AMPARs on postsynaptic membranes and the subsequent AMPAR-mediated synaptic transmission (Zeng et al., 2019).

3.2. CaMKII shuttling within ePSD

Another major class of proteins concentrated at ePSDs are enzymes and their substrates. CaMKIIα is the most abundant protein in ePSDs and is a kinase that modulates the phosphorylation status of many other PSD proteins (Bayer and Schulman, 2019; Hell, 2014; Herring and Nicoll, 2016; Lisman et al., 2012). It oscillates between different conformational states depending on the presence or absence of Ca^{2+}, which alters its binding partners (Baucum et al., 2015; Bayer et al., 2001; Perfitt et al., 2020) (Fig. 3B and C). CaMKIIα, in the absence of Ca^{2+} (autoinhibited state), binds to Shank3 and undergoes phase separation when in vitro purified proteins were mixed at physiological concentrations (Cai et al., 2021). In the presence of Ca^{2+}-CaM, autophosphorylation of CaMKIIα releases its autoinhibitory segment and thus allows its interaction with GluN2B. GluN2B binding disperses CaMKIIα condensation with Shank3, and instead, CaMKIIα is now enriched into the PSD-95−GluN2B condensates (Fig. 3C). The condensed CaMKIIα phosphorylates other PSD scaffold proteins including GKAP and Homer, subsequently promoting the enrichment of PSD-95 into phase condensates via enhanced interaction with phosphorylated GKAP (Zhu et al., 2017). This significantly enhances phase separation of the reconstituted ePSD (Cai et al., 2021), analogous to the structural LTP process in neurons in response to Ca^{2+} influx (Bosch et al., 2014; Lee et al., 2009; Matsuzaki et al., 2004). CaMKIIα can therefore shuttle between the PSD layer closer to the membrane where it senses Ca^{2+} signal and binds to the receptor tail, leading to the activation of its kinase activity, and the lower layer away from the Ca^{2+} signal where it stays in an autoinhibited conformation to be "stored" at PSD (Fig. 3C). These findings in vitro are consistent with observations made in situ from immunogold EM studies showing a reversible translocation of CaMKIIα from PSD pallium to PSD core upon stimulation (Dosemeci et al., 2016; Tao-Cheng, 2020).

3.3. Open questions

Biomolecular condensation via LLPS occurs above a threshold concentration. The condensed phase results in a massive concentration of protein constituents within it, while proteins can still freely move into or escape from the condensates. These unique properties of condensates might provide explanation for how ePSD assemblies are stably maintained in synapses and at the same time are subject to dynamic modulation during synaptic stimulation. Another distinct feature of phase separated biomolecular condensation is that the concentration of condensate constituents remains almost constant once the threshold concentration for phase separation is reached (Zeng et al., 2018). This observation predicts that a further increase of component concentration in the system would lead to a volume increase of the condensed phase. Indeed, overexpression of PSD scaffold proteins in neurons is often correlated with an enlarged spine head volume and an increased PSD area. On the other hand, removal of PSD scaffold proteins leads to shrinkage in spine head size (Zhu et al., 2016, 2017).

Synapses are highly plastic in response to various stimulations. Post-translational modifications are believed to play crucial roles in modulation of PSD assemblies during synaptic plasticity. Recent work illustrated how activity-dependent modifications by CaMKIIα, the most abundant kinase in ePSD, regulate phase separation in the system (Cai et al., 2021; Hosokawa et al., 2021). Palmitoylation of PSD-95 was reported to be essential for its postsynaptic localization and nanoclustering (Fukata et al., 2013; Jeyifous et al., 2016; Sturgill et al., 2009) and for its association with raft-promoting lipids (Tulodziecka et al., 2016). How does palmitoylation contribute to ePSD phase separation? Lipid phase separation in membranes has been an area of long-standing interests. How do the two phase-separated systems, lipid rafts and PSD condensates, communicate with each other? This is another important direction to follow in the future.

4. *Trans*-synaptic alignment of pre and postsynaptic termini via the synaptic cleft molecular assembly

There is now mounting evidence to suggest that the assembly of pre and postsynaptic scaffold structures is driven by LLPS (Fig. 1). How about the synaptic cleft? In the earliest EM studies, electron-dense material was already observed between the opposed synaptic membranes (Gray, 1959). Further studies revealed the existence of bridging fibrils spanning across the cleft intertwined with fibril-like structures running parallel to the synaptic membranes (Ichimura and Hashimoto, 1988). The density within the cleft is not uniformly organized but increases towards the membranes (Burette et al., 2012; Perez de Arce et al., 2015). At the edges of the synapse, pre- and postsynaptic membranes seem to make direct contacts with the cleft density to constrict a defined cleft space. These patterns of density suggest there might be subdomains within the cleft that could perform distinct functions in organizing synapses.

The cleft contains transsynaptic complexes that are arranged in periodic patterns and are highly laterally connected (Lucic et al., 2005; Zuber et al., 2005). Super resolution imaging analysis demonstrated the presence of nanocolumns across the pre- and postsynaptic termini, enabling neurotransmitter release and capture (Tang et al., 2016). Independent studies, using microscopic imaging approach combined with mathematical modelling, revealed that vesicle-membrane fusion sites are restricted to defined regions of the bouton membrane and the spatiotemporal distribution of release sites is activity dependent (Maschi and Klyachko, 2017; Tang et al., 2016). Photoactivated localization microscopy further confirmed that RIM density increased within 40 nm of these fusion sites (Tang et al., 2016). Using PSD-95 as an ePSD marker, Tang et al., measured densities of RIM and PSD-95 and revealed a clear spatial correlation between the components. Apart from the precise alignment of pre- and postsynaptic scaffolds, recent development of live gold labeling combined with functional EM further demonstrated that the readily releasable SVs in the active zone lie within 20–30 nm to the presynaptic Ca^{2+} channels and postsynaptic AMPARs (Brockmann et al., 2020). This *trans*-synaptic alignment, and subsequently the synaptic transmission, was lost when the presynaptic scaffolds, RIM and RIM-BP, were depleted, and could be restored by artificially linking the Ca^{2+} channel with the AMPAR. On the postsynaptic side, glutamate receptors are not uniformly distributed but exhibit differential localization patterns, with NMDARs clustered towards the central region and

AMPARs more to the edges or throughout the PSD depending on the type of synapses (Goncalves et al., 2020; Li et al., 2021; MacGillavry et al., 2013; Nair et al., 2013; Scheefhals and MacGillavry, 2018). What determines the segregation pattern of different receptors? Biochemical reconstitution using CaMKII, PSD-95, GluN2B_CT and TARP_CT demonstrated that PSD-95/TARP_CT form a 'core' surrounded by a CaMKII/GluN2B_CT-rich liquid 'shell', recapitulating the segregation of AMPAR and NMDAR nanodomains in synapses (Hosokawa et al., 2021). In addition, the cytoplasmic tail of neuroligin-1, a synaptic adhesion molecule, partitions into the PSD-95−TARP-CT phase condensates but separates from the CaMKII−GluN2B phase condensates. This observation fits well with the nanocolumn structure spanning the synaptic cleft. AMPARs have a lower affinity for glutamate than NMDARs (Lisman and Raghavachari, 2006), and the precise alignment of AMPARs to the neurotransmitter release sites via transsynaptic adhesion molecules can increase the efficacy of synaptic transmission (Biederer et al., 2017; Hruska et al., 2018; Scheefhals and MacGillavry, 2018; Tang et al., 2016). The observation of phase-in-phase nanodomain structures in vitro only starts to unravel the tip of the iceberg with regard to *trans*--synaptic organization, and many questions are awaiting to be addressed. How do synaptic adhesion molecules and receptors participate in condensate assembly across the synaptic cleft? How do the pre- and postsynaptic condensates communicate with each other? What role does the synaptic cleft play in aligning the pre-and postsynaptic condensates? These are certainly interesting directions for future investigations.

5. Conclusions and perspective

Our understanding of synaptic assembly and organization has rapidly increased over the past decade. Although the repertoire of proteins vital for pre- and post-synaptic structure assembly and function has been well documented, the organizational principles remained poorly understood. In particular, the observation of nanodomains formed by receptors and scaffold proteins could not be explained. Work in recent years has demonstrated that synaptic organization is underlined by LLPS, although we are just beginning to understand the biophysical rules governing this phenomenon (Chen et al., 2020). Phase separation is an inherent property of biopolymers including proteins and nucleic acids, and thus cares should be taken to characterize whether phase separation of purified biomolecules or their complexes can occur in vitro under conditions relevant to their physiological settings. When possible, it will be valuable to test whether phase separations observed for biomolecules or their complexes in vitro bear biological relevance in vivo (Feng et al., 2019a; McSwiggen et al., 2019). For instance, mCherry, a large fluorescent tag, alone was shown to form condensates and thus could introduce artificial impacts on the phase behaviour of the fusion protein (McDonald et al., 2020). EGFP, a weak dimer (Zacharias et al., 2002), was also shown to artificially lead to puncta formation in cells (Park et al., 2021), although the protein is widely used in numerous LLPS studies. It is therefore suggested to remove large protein tags in phase separation assays in vitro. Polymers such as PEG are frequently used as crowding reagents to trigger condensation in some experiments. However, it is questionable whether such reagents can truly mimic cellular environment especially in the presence of unusually high concentrations. Another common issue is nucleic acid contamination that is widely shown to promote protein phase separation. Rigorous controls need to be conducted before drawing conclusions. There remains much to learn about the structural complexity within synapses, especially now through the lens of phase separation phenomena. Do AZ and ePSD assemblies contain multiphase substructures? What properties drive phase immiscibility? How do AZ and ePSD condensates communicate with each other? Do protein complexes in synaptic cleft also participate in phase separation? How are nanocolumnar structures assembled and maintained across the synaptic cleft? In this review, we have mainly focused on the structural organization of excitatory synapses. The observation of inhibitory PSD (iPSD) area could be dated back to as early as 1950s (Gray, 1959), while recent cryo-EM tomography studies revealed that iPSD appears as a thin sheet-like structure in its native state (Liu et al., 2020; Tao et al., 2018). The major scaffold protein in inhibitory synapses, gephyrin, phase separates together with glycine or $GABA_A$ receptors both in solution and on supported membranes, suggesting that iPSD assembly is also underlied by LLPS (Bai et al., 2021). How do iPSD and ePSD condensates communicate with each other and thus determine the specific synaptic types and ultimately neuronal circuit balance? One might predict that PSDs in neuromuscular junction may also be formed by phase separation involving the key scaffold protein rapsyn.

In summary, the findings of phase separation-mediated formation and regulation of various synaptic assemblies in the past few years open many new research directions for us to better understand the relationship between synaptic nanostructures and functions. We anticipate many new discoveries will follow in the area connecting phase separation with synapse formation and function.

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