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Neuronal Synapses: Microscale Signal Processing Machineries Formed by Phase Separation?

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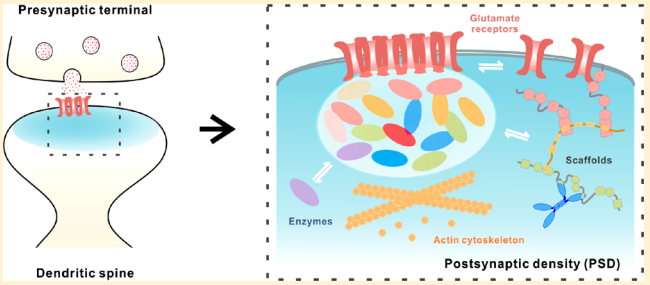
ABSTRACT: The organization principles underlying non-membrane-bound organelles have started to unravel in the past 10 years. A new biophysical model known as biomolecular condensates has been proposed to explain many aspects of membraneless organelle assembly and regulation. Neurons are extremely complex, and each neuron can contain tens of thousands of synapses, building an extensive neuronal circuit. Intriguingly, neuronal synapses are characterized by specialized compartmentalization, where highly enriched supramolecular complexes are semi-membrane-enclosed into submicrometer-sized signal processing compartments. Recent findings have demonstrated that this postsynaptic density may be driven by phase separation, and an increasing number of studies of membraneless compartments have shed light on the important molecular features shared by these organelles. Here, we discuss the unique morphology and composition of synapses and consider how synaptic assembly might be driven by phase separation. Understanding the molecular behavior of this semi-membrane-bound compartment could ultimately help to explain the mechanistic details underlying synaptic transmission and plasticity, as well as the numerous brain disorders caused by synaptic defects.



The human brain comprises nearly 100 billion neurons, wired to build extensive neural circuit networks. Ramon y Cajal first made his beautiful drawings of individual neurons more than a century ago, leading to the realization that information could be transmitted through neuronal networks in the brain. In particular, he observed that neural cells are not continuous but rather contiguous with gaps between them.[1] Such micrometer-sized junctions were later termed synapses by Sherrington.[2] Synapses are now known to be the primary sites for information transmission and processing in the central nervous system, ultimately controlling brain function.

Synapses are not static machines, simply passing information encoded by action potentials from one neuron to another. Instead, they are extremely plastic. At the presynaptic termini, vesicles packed with neurotransmitters are transported to, and fuse with, the presynaptic membranes, where the neurotransmitters are released into the synaptic cleft. These signaling molecules then bind to and activate the transmembrane receptors at the postsynaptic termini. This process subsequently triggers the recruitment and activation of downstream signaling pathways. The plasticity of this process is evident in the significant changes in morphology and protein content that take place in response to various neuronal activities on relatively short time scales. During long-term potentiation (LTP), postsynaptic depolarization unblocks NMDA-type glutamate receptors. This opens transmembrane channels, leading to an increased influx of Ca^{2+} ions that activate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). This kinase phosphorylates its target proteins and triggers downstream signaling pathways, leading to the growth of the actin cytoskeleton and the recruitment of AMPA receptors (AMPARs).[3−6] During LTP, the neck of dendritic spines (which connect the head to the shaft of these structures) becomes wider and shorter, while the volume of the spine head increases.[7] Such morphological changes facilitate the transport of synaptic proteins from the dendrites into the spines. These changes lead to an increased efficacy of synaptic transmission that can last from hours to days. Long-term depression (LTD) is the opposite of LTP. During LTD, AMPARs are removed from the postsynaptic membrane.[8] The downstream signaling pathways are hampered, and the actin cytoskeleton content is diminished.[9,10] The spine head shrinks, and many synaptic components are removed.[11,12] These activity-dependent chemical and structural modifications lead to the remodeling of neuronal networks and subsequently control emotion, learning, and memory. Impairments in synaptic plasticity have been implicated in a broad range of psychiatric disorders such as intellectual disorders, autism, and schizophrenia.[13−19]

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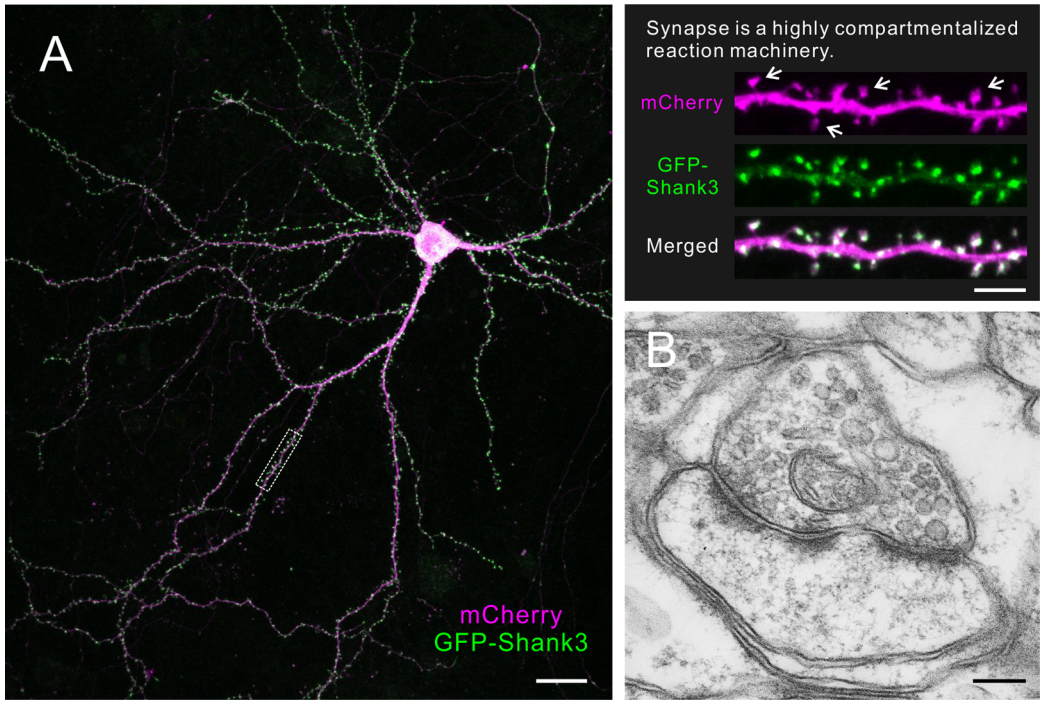

Figure 1. Illustration of neuronal morphology and an excitatory synapse. (A) Confocal image of a mice hippocampal pyramidal neuron labeled with GFP-Shank3 and mCherry cell fill. The scale bar is 20 μm. A close-up view of a segment of a dendritic branch (dotted box) is shown at the top right. In this expanded view, one can clearly observe that the compartmentalized protrusions that are away from the dendritic shaft are enriched with GFP-Shank3. The scale bar is 4 μm. (B) EM image of a perforated synapse. The presynaptic terminal contains synaptic vesicles, opposing the postsynaptic density. PSD refers to the layer of electron-dense material beneath the postsynaptic membrane bilayer. The scale bar is 200 nm. Image by SynapseWeb, Kristen M. Harris, PI, http://synapseweb.clm.utexas.edu/. Copyright 1999–present by Dr. Harris, all rights reserved.

Understanding the molecular mechanisms behind synaptic transmission and plasticity is essential to understanding normal and diseased brain functions.

Underneath the postsynaptic membrane lies an electron-dense material known as the postsynaptic density (PSD).[20,21] The PSD concentrates hundreds of proteins, including adhesion molecules, transmembrane receptors and channels, scaffolding and adaptor proteins, and actin cytoskeletal components.[22−25] In addition, small GTPases and their modulators, as well as signaling molecules such as kinases and phosphatases, are also found within the PSD.[23] Synaptic plasticity is closely linked to the number of glutamate receptors across the membrane and the area of the PSD underneath it.[5,7,26−28] Therefore, since the discovery of the PSD, its assembly and regulation have been a main focus of synaptic research. Our understanding of PSD components and their interaction networks has rapidly improved over the past few decades. Many proteins required for PSD organization have been captured at atomic resolution, and we are beginning to link structural information to function. However, some of the most fundamental questions remain unanswered. For instance, unlike the classical membrane-bound organelles, synapses show a very specialized compartmentalization. Each synapse can have multiple protein-dense assemblies that are not enclosed by lipid membrane bilayers. Neighboring synapses can make distinct morphological changes in response to different stimuli, on a time scale of a few seconds.[5,7] How is the PSD stably assembled underneath the postsynaptic membrane? How can a synapse undergo distinct morphological and molecular changes on a time scale of a few seconds but hardly affect neighboring synapses? How is the sharp concentration gradient between the self-assembled protein cloud in the PSD and the much more diluted pool in the cytoplasm maintained, given that there is no physical barrier between them? How can molecules specifically and freely enter or escape the PSD within a short time window during neuronal plasticity?

In this review, we first briefly discuss the unique structural features of neuronal synapses and present the biochemical and cellular evidence leading to the new conceptual model of phase separation-driven PSD assembly. It is noted that phase separation also occurs in early neurogenesis[29] and has been implicated in a range of neurodegenerative disorders.[30−35] In this review, we decided to focus on synaptic physiology. Recent studies of non-membrane-bound organelles have unified many structural features of the proteins involved in such a biophysical model. On the basis of these features, we suggest a potential mechanism underlying PSD assembly on a global scale. Finally, we discuss the biological importance of phase separation-driven PSD assembly in synapses.

SEMI-MEMBRANELESS PSD ORGANIZATION

Cells organize macromolecules into specialized compartments for proper cellular functioning. Such compartmentalization can be achieved via the formation of membranes, as is the case in mitochondria, lysosomes, and the endoplasmic reticulum (ER). Membranes serve as a physical barrier to localize and concentrate proteins of interest, control local conditions, and confer organelle specificity. However, some cellular compartments are not enclosed by a membrane, for example, the nucleoli in the nucleus, P granules in germ cells, centrosomes, and the synapse in neurons.[20,36−39] Take, for example, the mouse hippocampal pyramidal neurons (Figure 1). Each cell contains numerous submicrometer-sized protrusions/synapses along dendrites responsible for receiving signals released by the

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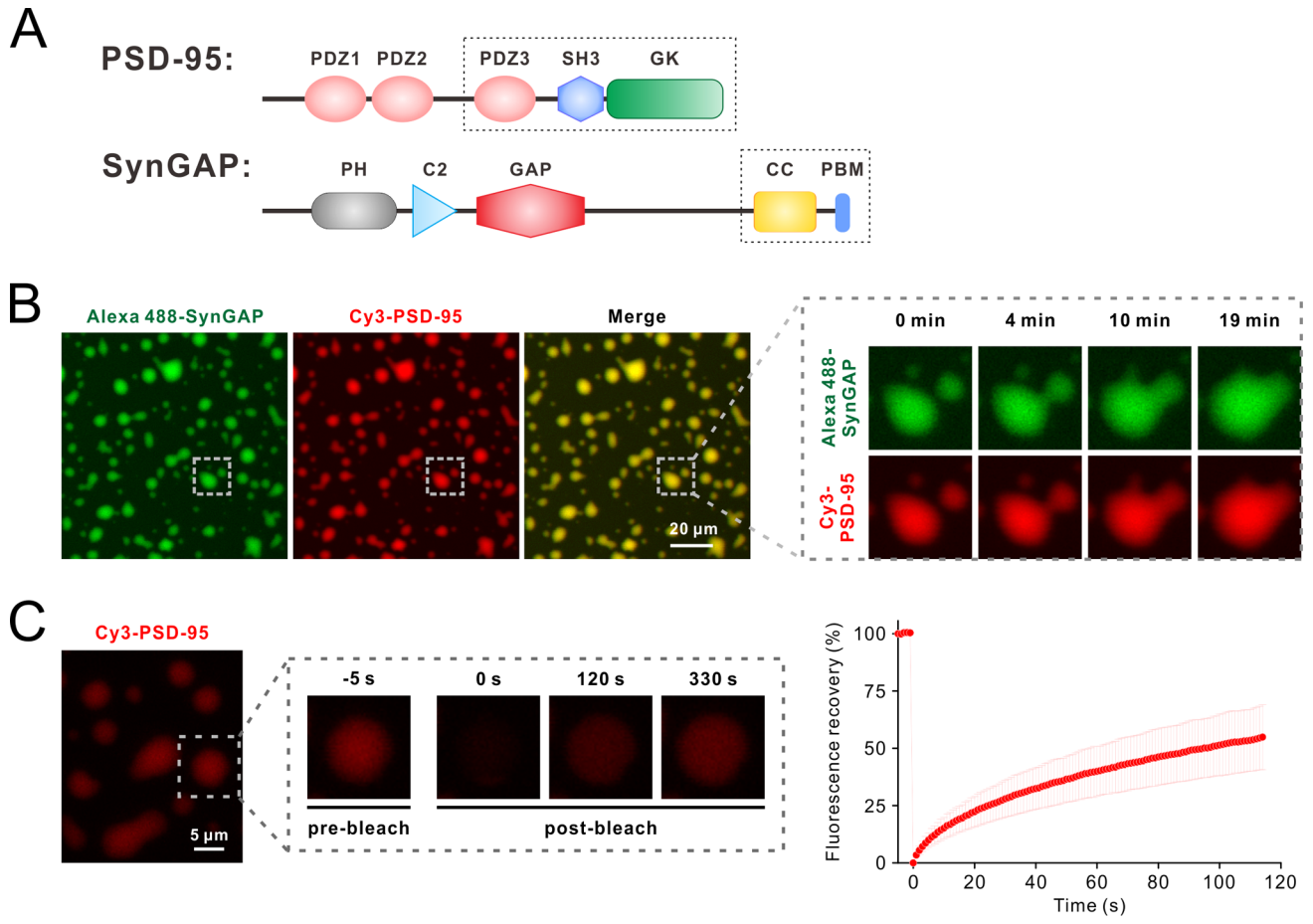

Figure 2. PSD-95 and SynGAP undergo LLPS *in vitro*. (A) Schematics of PSD-95 and SynGAP showing protein domain organizations. Dotted boxes highlight the constructs used in subsequent *in vitro* experiments. (B) Co-localization of SynGAP and PSD-95 in the liquid droplets. Fluorescently labeled proteins were mixed at a 1:1 molar ratio at 50 μM in 50 mM PBS (pH 7.4), 100 mM NaCl, and 5 mM DTT. Imaging was performed in a flow chamber at room temperature with a Zeiss LSM 880 confocal microscope. The enlarged images show fusion between smaller droplets upon contact. Images taken from ref 39 with permission. Copyright 2016 Elsevier Inc. (C) FRAP analysis of Cy3-PSD-95 in condensed droplets showing its free exchange with the dilute aqueous phase. The highlighted box shows representative images of time-lapse fluorescence recovery from Cy3-PSD-95 after photobleaching. The graph on the right shows fluorescence recovery over time (20 droplets analyzed). Error bars indicate the standard deviation. Images taken from ref 39 with permission. Copyright 2016 Elsevier Inc.

axon terminals of interacting neurons (not visible by current staining methods). Electron microscopy (EM) studies first observed the presence of an electron-dense thickening underneath the plasma membrane of each excitatory synapse, known as the PSD (Figure 1B). Intriguingly, the PSD is semi-membraneless, with its top side attaching to the postsynaptic membrane bilayer while the bottom side is in free contact with the cytoplasm. Early biochemical and EM studies have established that the PSD is densely packed with proteins,[22,40−42] and recent proteomic analyses have further confirmed the presence of thousands of different proteins with distinctive abundances at the PSD.[23,43] Synaptic plasticity is tightly coupled with the precise removal or addition of PSD components on a time scale that can be as fast as a few seconds. It is imperative to understand how these processes are regulated without the aid of plasma membranes, as is the case in classical organelles. Synapses can be excitatory or inhibitory. However, the inhibitory synapse contains a much less obvious electron-dense thickening beneath the plasma membrane, although extensive protein interaction networks have also been identified [termed inhibitory PSD (iPSD)]. In this review, we focus on excitatory PSD (ePSD) and its potential mechanism of assembly and regulation.

PHASE SEPARATION-DRIVEN PSD ASSEMBLY

For decades, little progress was made toward understanding the assembly of non-membrane-bound organelles. A conceptual breakthrough was made when P granules in the germ cells of *Caenorhabditis elegans* were shown to be condensates that phase transit from the surrounding cytoplasm.[36] P granules exhibit liquid-like properties. (1) They fuse with one another upon contact. (2) They show a spherical morphology that deforms upon contact with other surfaces. (3) They show shear stress-induced flowing and dripping behaviors. With the appreciation of this new physical model, extensive studies of membraneless organelles have been performed in the past 10 years. These works are discussed extensively in several recent reviews,[44−47] and here we focus on excitatory synaptic signaling complex assembly and regulation.

PSD Assembly Might Be Driven by Phase Separation. A recent study from our lab demonstrated that *in vitro*-purified PSD-95 PDZ-SH3-GK (PSG) tandem and SynGAP coiled-coil (CC) PDZ binding motif (PBM) undergo liquid−liquid phase separation (LLPS) in solution and in cells. PSD-95 and SynGAP are two very abundant proteins that localize at the PSD of excitatory synapses.[48] Genetic studies of human

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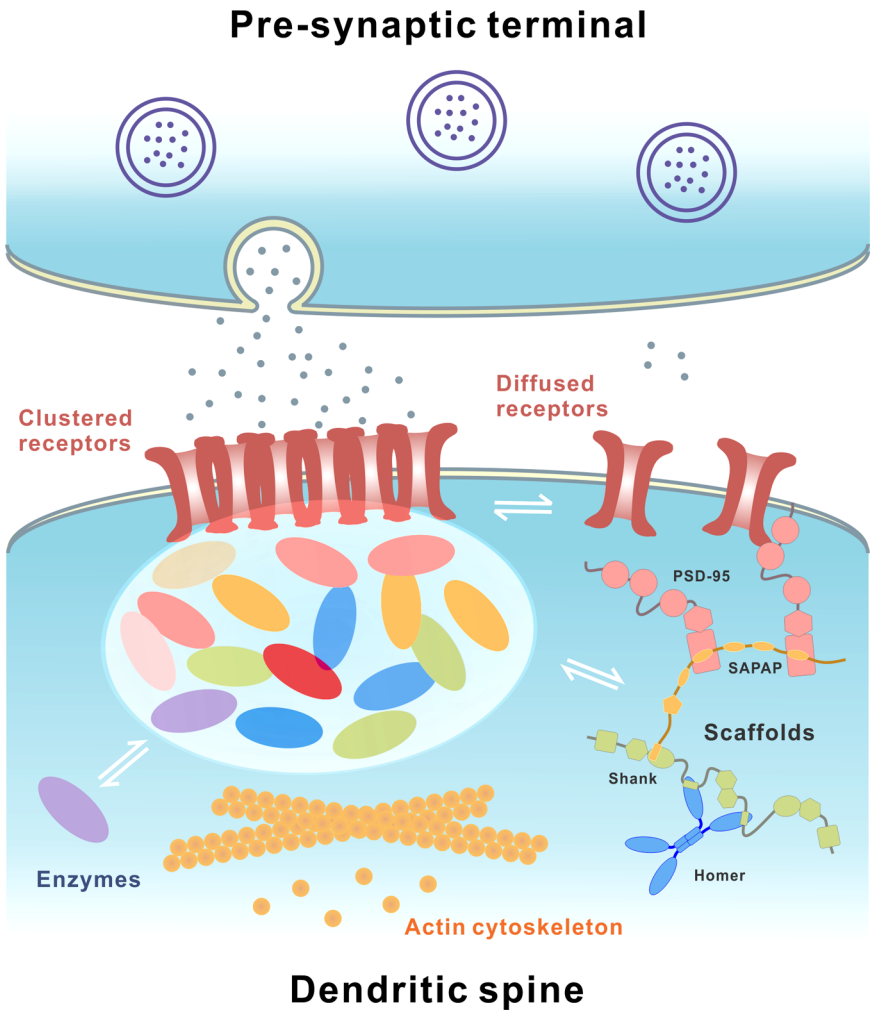

Figure 3. Potential mechanistic basis of phase separation-driven PSD assembly. At the presynaptic terminus, vesicles packed with neurotransmitters are transported to, and fuse with, the presynaptic membrane, where the signal molecules are released. Neurotransmitters bind to and activate the transmembrane receptors at the postsynaptic membrane. Receptors can cluster into nanodomains, which was shown to significantly enhance the efficiency of signal transmission. At the PSD, scaffold proteins, in particular, PSD-95, SAPAP, Shank, and Homer, form layered interaction networks. These scaffold proteins contain multiple interaction domains connected by unstructured loop regions between them. Many of them show a propensity to undergo multimerization in solution. Their intrinsic features lead to the hypothesis that PSD scaffold proteins might form biomolecular condensates to recruit and enrich many other PSD components such as enzymes and the actin cytoskeleton. Note that the domain structures are not drawn to scale in this figure.

patients with brain disorders have revealed a spectrum of mutations in both proteins.[13,49,50] PSD-95 is a member of the membrane-associated guanylate kinase (MAGUK) superfamily and is characterized by the presence of three N-terminal PDZ domains followed by a SH3 domain and a GK domain (Figure 2A). In mammals, PSD-95 has three other homologues: PSD-93, SAP97, and SAP102. Together, they are the major scaffolding proteins that underlie the PSD architecture and regulate synaptic strength and transmission.[51−53] SynGAP stimulates the GTPase activity of Ras/Rap proteins and hence acts as a negative regulator of the excitatory synaptic activity (Figure 2A).[54−57] During and after LTP, SynGAP quickly disperses from the PSD, which in turn triggers Ras/Rap activation and a series of downstream signaling events. This eventually leads to enlarged spine sizes and enhanced synaptic strengths.[58−60] When purified SynGAP CC-PBM was mixed with PSD-95 PSG tandem[39] or full-length PSD-95 (unpublished data), protein condensates were readily observed via light microscopy (Figure 2B). These condensates are spherical, showing a range of diameters, and can coalescence into larger droplets upon contact. Fluorescence recovery after photobleaching (FRAP) analysis of the fluorescently labeled components showed constant exchange of molecules with the surrounding aqueous solution, further confirming their liquid-like properties (Figure 2C).[39] The condensate assembly is interaction- and oligomerization-dependent. Mutation of the PBM−PDZ interaction interface (in SynGAP CC-PBM) prevents LLPS *in vitro*, suggesting the specificity of this biochemical reaction.[39] However, a single intermolecular interaction site is not sufficient to promote phase separation. SynGAP CC-PBM forms a parallel coiled-coil trimer, and this multimerization is essential for LLPS, as its monomeric mutant fails to form phase condensates in solution even though binding affinity is retained.[39] In neurons, either mutation on SynGAP significantly reduces the extent of SynGAP localization at the PSD. In addition, these mutants show a much larger proportion of synaptic dispersion upon chemLTP stimulation. The spine head is massively enlarged when endogenous SynGAP is replaced with its monomeric mutant in the basal state, likely representing a hyperactivated state.[39] Upon chemLTP

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induction, the mutant neurons also display much larger spine head sizes and more receptor recruitment to the synapse. Interestingly, the mutant neurons are also sensitive to a much weaker chemLTP induction (20-fold less glycine stimulation), which is not capable of inducing LTP in neurons containing the wild-type SynGAP.[39] These observations strongly suggest that phase separation might be the mechanism for semi-membrane-bound PSD organization in synapses.

PSD Fulfills the Criteria of Phase Separation. The PSD contains supramolecular complexes that separate from the surrounding homogeneous solution state, despite the lack of a physical barrier. Proteins within this condensate can be exchanged with its cytoplasmic fraction, as shown by FRAP analysis in cultured neurons.[61−64] Proteins also undergo internal rearrangement within the PSD. Using single-particle tracking, where the selected protein is labeled with a photoactivatable fluorescent tag, the trajectories of PSD proteins can be followed. It was noticed that many proteins show random yet confined movement within the PSD.[65−68] Photoactivated localization microscopy (PALM) studies in living neurons demonstrated that four major PSD scaffold proteins (PSD-95, SAPAP, Shank, and Homer) form subsynaptic clusters.[69] Strikingly, these scaffold clusters continuously reorganize over time, especially during LTP and LTD. Therefore, PSDs in neurons have all the features of protein condensates formed by phase separations.

PHASE SEPARATION DRIVEN BY MULTIMODULAR DOMAIN INTERACTIONS

Since the first study showing that P granules in germ cells assemble via LLPS *in vivo*, the list of non-membrane-bound organelles that are organized by this biophysical model has been ever growing. Systematic analysis of the molecules involved in phase separation and their reaction modes has led to the realization that they share certain features. This led to the new name of biomolecular condensates to designate non-membrane-bound supramolecular complexes that form via phase separation.

Biomolecular condensates are often driven by multivalent interactions. In the dilute solution phase, molecules always exhibit weak interactions with the solute and nonspecific interactions with themselves. However, the intermolecular interactions are much weaker and hence less energetically favorable than those with the solute molecules. In the presence of specific macromolecular interactions, that is, ligand binding or oligomerization, the macromolecular interactions become sufficiently stronger than the macromolecule−solute interactions, and this changes the phase behavior and/or solubility of the mixture. Increases in binding affinity between the molecular modules and the number of these modules promote polymer assembly and eventually lead to phase separation at its solubility limit.[46,70] Molecules then assemble into condensates that demix from the surrounding aqueous phase. The liquid phase droplets assembled from PSD-95 PSG tandem and SynGAP CC-PBM provide a good example of this basic organization principle. Disruption of multivalency, by either knocking out the interaction interface or abolishing the SynGAP trimer assembly completely, prevented the assembly of phase condensates. It was noted that the concentration-dependent PSD-95 PSG/SynGAP CC-PBM phase transition occurred at a very high concentration, likely above the physiological values. Nonetheless, the PSD has long been known to be a signaling machinery driven by multivalent interactions.[53] Here we briefly discuss the modular interactions between key PSD proteins. We speculate that the presence of multiple modular interactions may significantly lower the phase separation threshold and that the PSD may be another example of a biomolecular condensate.

Multivalency among PSD Scaffold Proteins. The PSD concentrates and organizes thousands of proteins, serving as a signaling machinery in response to synaptic activities. PSD scaffold proteins are highly abundant and provide a binding platform for various other components (Figure 3). The abundant MAGUK family proteins lie close to the postsynaptic membrane. They are characterized by the presence of multiple PDZ domains, an SH3 domain, and a GK domain.[53] These domains are all known to be protein interaction modules and hence make MAGUKs well suited to being scaffold proteins.[25,71,72] The relationship between multivalency and phase separation was demonstrated using engineered poly-Src homology 3 (polySH3) proteins and polyproline-rich motif (polyPRM) ligands that carry different numbers of copies of the interaction module.[70] The study concluded that the valency and affinity of interaction modules are key factors of phase transition. In the PSD, most of the PDZ domain scaffold proteins contain multiple PDZ domains [e.g., each discs large (DLG) MAGUK contains three PDZs, the glutamate receptor interacting protein (GRIP) contains seven PDZs, and the AMAPR binding protein (ABP) contains six PDZs]. Such polyPDZ organization provides a perfect platform for binding and localizing many other PSD proteins such as synaptic transmembrane proteins. Glutamate receptors and synaptic adhesion molecules mostly possess PBMs. Interactions between these PBM and PDZ domains are known to be essential for the targeting, clustering, and removal of the synaptic transmembrane proteins during LTP.[25,73,74]

In addition to the upper PSD layer, multivalent scaffold molecules are also found in the intermediate and cytoplasm-facing regions. For instance, SAPAP family proteins contain multiple (two to five) guanylate kinase (GK) binding repeats (GBR) that can serve as the binding platform for the GK domain containing proteins such as PSD-95.[75] The PBM of SAPAP is known to interact with the PDZ domain of Shank that lies toward the cytoplasmic face of the PSD.[76] Shank has a C-terminal sterile alpha motif (SAM) domain that is involved in protein oligomerization.[77,78] Multimeric Shank forms higher-order complexes, with tetrameric Homer assembling into matrix-like structures, as illustrated by negative staining EM.[79] This complex formation depends on Shank−Homer interaction and the multimerization of both proteins.

In addition to the modular domains, PSD scaffold proteins also contain a large proportion of unstructured regions that could provide multiple weakly adhesive modules.[33,46,80,81] For example, apart from its C-terminal helical GKAP homology domain, the whole of the SAPAP protein is highly intrinsically disordered. The evolutionarily conserved Shank proteins also contain a long unstructured region between their N- and C-terminal domains.

Multivalency among Client Proteins. Enzymes and modulatory proteins are concentrated at the PSD via scaffolding components (Figure 3). SynGAP/PSD-95 phase separation provides an example of enzymes that can be enriched in the condensed phase. CaMKII, the most abundant protein at the PSD, is a calmodulin-dependent protein kinase whose activation is triggered by Ca^{2+} influx during LTP.[82] CaMKII has four isoforms, and its subunits assemble into dodecameric holoenzymes arranged as two stacked rings.[83−86]

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Interestingly, subunits of CaMKII might mix between isoforms to form a heteromeric holoenzyme, further increasing the structural complexity and subsequently its functional diversity.[87−89] GBR in SAPAP can be phosphorylated by CaMKII *in vitro* and in cultured neurons.[90−92] Recently, it was shown that each GBR contains a conserved phosphorylation motif that might be the substrate sequence for other PSD kinases.[93] Phosphorylation on GBR dramatically increases its binding affinity for PSD-95. The presence of a conserved phosphorylation motif in each copy of GBR in SAPAP proteins may increase multivalency and subsequently promote phase separation. Mass spectrometry analysis has mapped extensive numbers of phosphorylation sites on PSD proteins.[90] Intriguingly, a large proportion of these sites are localized within unstructured yet highly conserved regions. We speculate that phosphorylation may change surface charges of substrate proteins or lead to conformational changes that can modulate the accessibility of the multivalent regions or tune the bindings among PSD proteins and thereby regulate PSD condensate formation.

The dendritic spine is enriched with actin. Importantly, the activation and removal of glutamate receptors, in response to neuronal activities, are closely related to actin dynamics. For instance, activation of NMDA receptors (NMDARs) induces spine outgrowth and enlargement via actin-dependent mechanisms,[94] whereas actin depolymerization leads to the loss of synaptic NMDAR or AMPAR accumulation.[95−97] Therefore, many actin cytoskeleton proteins are also found at the PSD, such as Cortactin and the Arp2/3 complex. A recent study investigated the actin regulatory network formed of nephrin, Nck, and neural Wiskott-Aldrich syndrome protein (N-WASP).[70,98] It illustrated that the supramolecular complex formed by these three proteins significantly enriches actin bundles in the presence of N-WASP target protein and the Arp2/3 complex. N-WASP is also recruited to the PSD via SH3−PRM binding. Cortactin is another actin regulatory protein that binds to the polyP sequence in Shank.[77] Are these actin regulatory proteins also enriched in the PSD via phase separation? How could this enrichment change actin dynamics?

FUNCTIONAL IMPLICATIONS

Biomolecular condensates seem to be a common theme for the organization of non-membrane-bound organelles. Earlier in this review, we discussed the properties of some of the major PSD proteins and their propensity to form phase condensates, given the extensive networks of intermolecular interactions and self-interactions. Here we discuss how the properties of phase separation might be reflected in biological functions in neurons and in other cell types in general.

Phase separation occurs above certain concentration thresholds, and once this threshold is reached, proteins in the condensed phase are highly enriched and stably assembled. These components can still freely exchange material with the surrounding solution. If the PSD were assembled as a homogeneous solution state, concentration-dependent protein interaction would mean that more protein needed to be synthesized to reach concentration-dependent diffusions. This consumes both energy and material, especially in synapses where numerous PSDs can assemble autonomously in a single neuronal cell (e.g., one human purkinje neuron can have >150000 excitatory synapses/PSDs). In addition, many of the known protein−protein interaction strengths (e.g., PDZ−PBM bindings) are on the scale of micromolar or even weaker. However, this is not a significant problem in phase separation-driven assembly. Biomolecular condensate formation can be promoted by the presence of multiple modular interactions and weak protein−protein interactions, even though the individual binding may not be particularly strong.

The assembly of the condensed phase also confers reaction specificity. For example, reconstituted T cell receptor signaling clusters concentrate kinases but exclude phosphatases to stabilize the phosphorylation-dependent condensates.[99] In neurons, synapses can be excitatory or inhibitory. There are a large number of inhibitory synapses on dendrites, and in many cases, inhibitory synapses coexist with excitatory synapses on dendritic spines.[100,101] Interestingly, inhibitory and excitatory synapses do not physically intermix despite being within a single spine with a volume of <0.1 fL. How is this achieved, given that proteins in different signaling pathways are present in the same tiny compartment within a dendritic spine? Do ePSD and iPSD share the same phase separation-conferred selectivity as seen in the T cell system?

Synapses are highly plastic in response to neuronal activities. They need to undergo compositional and structural rearrangements within as little as a few seconds. Phase separation provides a substantial advantage, because this feature can be switched on and off by controlling the formation and dispersion of a condensed phase. The condensed phase is sensitive to post-translational modifications such as phosphorylation.[31,99,102] Small changes in the relative stoichiometry of scaffold components can also shift condensed phase formation.[46] Recently, it has been shown that phase transition is often sensitive to pH or salt concentration changes of protein mixtures.[81,103,104] Considering that neurotransmitter-triggered synaptic stimulations are always coupled with ion fluxes or pH changes in the highly localized, tiny compartments of dendritic spines, can PSD phase transition formation or dispersion be regulated by changes in ion concentration or pH in living neurons?

CONCLUSION AND PERSPECTIVES

Research in the past 10 years has greatly expanded our understanding of the organization and function of membraneless organelles. Neuronal synapses inherit a unique compartmentalization in which thousands of proteins are concentrated in an electron-dense zone called the PSD. The disc-shaped PSD visible under electron microscopes is attached to the postsynaptic plasma membrane on one side and open to the cytoplasm on the other side, and for decades, it was not understood how such self-assembled protein complexes could be assembled, maintained, and disassembled. The observation that two abundant PSD proteins, PSD-95 and SynGAP, form phase separation condensates has opened a new research area in the synaptic biology field. However, this is only the beginning, and many questions remain unanswered. Can we expand the *in vitro* reconstitution system to a more global interaction network by including other multivalent PSD scaffold proteins? Distinct from many other phase separation systems in which multivalency is largely determined by intrinsically disordered protein segments,[33,80,81,105−108] the PSD contains extensive networks of intermediate to strong protein−protein interactions that may drive biomolecular condensate formation. Does this high assembly specificity provide a higher specificity for regulation during neuronal activities? An *in vitro*-reconstituted system would provide opportunities to investigate the enrichment and function of

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client molecules such as transmembrane receptors and kinases. Glutamate receptors are found to cluster into nanodomains at excitatory synapses.[53,67,69] Is this receptor clustering promoted by phase separation of scaffold proteins? Does the size of phase condensates at the postsynaptic side help define the boundary and hence the alignment of presynaptic termini or vice versa? Biochemically purified PSD shows extremely stable, hydrogel-like, and detergent-resistant properties because of the complicated cross-linking interactions among many PSD proteins. In the presence of extensive multimodular interactions, would previously observed liquid droplets undergo a transition into gels or glass-like structures *in vitro* and in living neurons? EM and super-resolution microscopic studies showed that the PSD scaffold proteins form a layered organization that is able to recruit and localize many other components.[109−112] How is this underlying order achieved and maintained in a phase separation system? Intriguingly, recent studies in stress granules and nucleoli suggest that the condensed phase can contain multiple distinct subcompartments.[113,114] Is such a phase in phase phenomenon underlying the geometrical organization of the PSD scaffolds?

In this review, we have largely focused on the PSD at excitatory synapses, but we do speculate that the presynaptic active zone and other types of synapses might also be organized by biomolecular condensates. EM studies of presynaptic termini did reveal a layer of electron-dense material close to the plasma membrane, later known as dense projections, if synapses were properly fixed.[115,116] Interestingly, the core constituents of the active zone also exhibit a multimodular domain organization and a large proportion of unstructured regions.[117] It is therefore possible that active zone proteins may assemble into condensed supramolecular complexes via multivalent interactions and oligomerization. Finally, it should be emphasized that information derived from *in vitro* reconstitution systems should be combined with *in vivo* studies to elucidate the role of individual proteins at the PSD and eventually to improve our understanding of the molecular mechanism of synaptic assembly, maturation, and function.

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Notes

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