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# Phase separation as a mechanism for assembling dynamic postsynaptic density signalling complexes

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The postsynaptic density (PSD) is an electron dense, semi-membrane bound compartment that lies beneath postsynaptic membranes. This region is densely packed with thousands of proteins that are involved in extensive interactions. During synaptic plasticity, the PSD undergoes changes in size and composition along with changes in synaptic strength that lead to long term potentiation (LTP) or depression (LTD). It is therefore essential to understand the organization principles underlying PSD assembly and rearrangement. Here, we review exciting new findings from recent *in vitro* reconstitution studies and propose a hypothesis that liquid–liquid phase separation mediates PSD formation and regulation. We also discuss how the properties of PSD formed via phase separation might contribute to the biological functions observed from decades of researches. Finally, we highlight unanswered questions regarding PSD organization and how *in vitro* reconstitution systems may help to answer these questions in the coming years.

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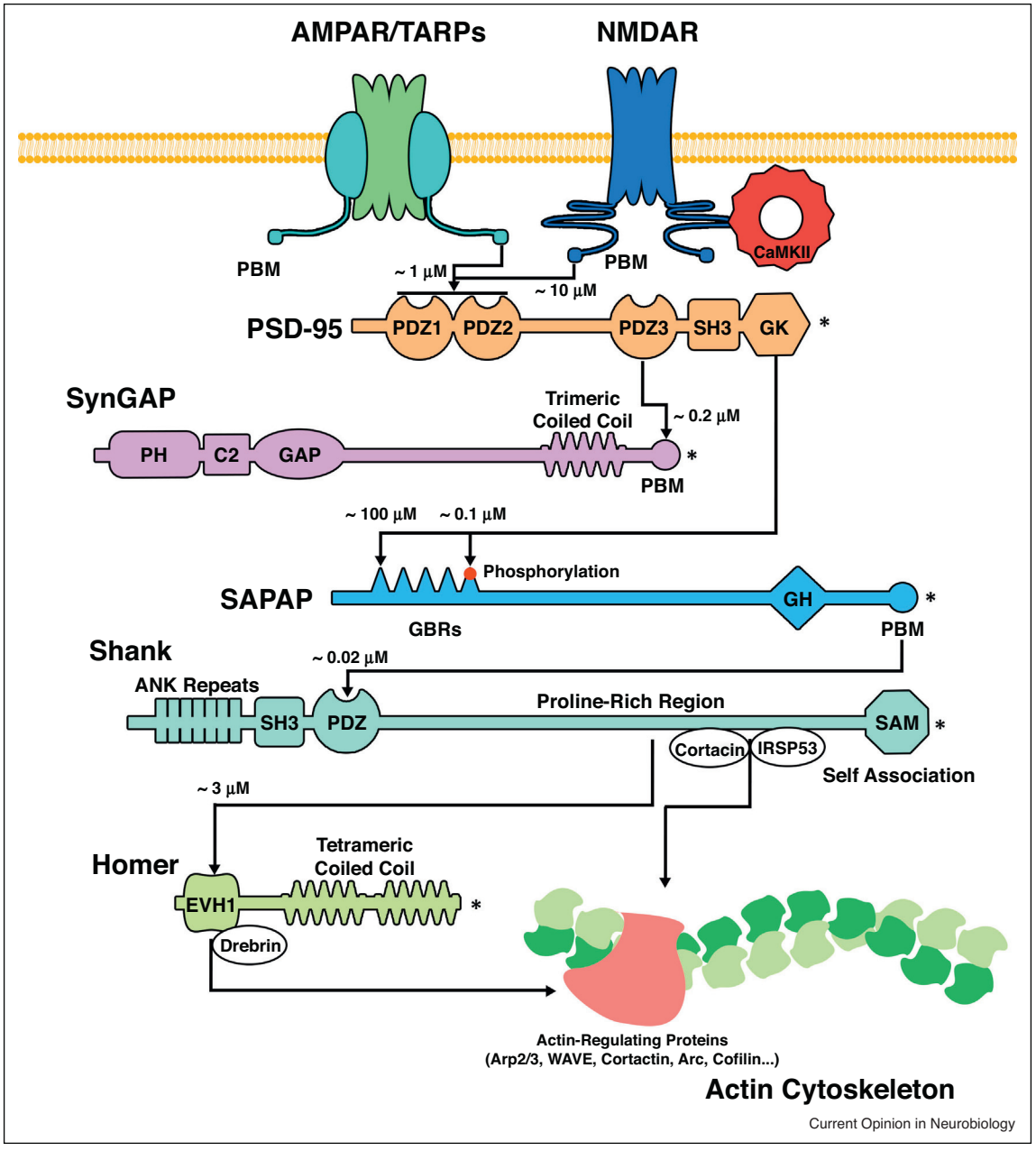
## Introduction

The human brain is extremely complex with one hundred billion neurons wiring together via the connecting nodes known as synapses to form functional circuits. Synapses serve as communication hubs for information transmission and processing in the central nervous system, ultimately controlling brain function. Therefore, understanding synaptic formation and regulation is essential for understanding how a neural circuit is built to perform discrete activities. Synapses can be excitatory or inhibitory depending on the type of neurotransmitters received at the postsynaptic termini. Beneath postsynaptic membranes lies a layer of electron dense material known as the postsynaptic density (PSD). Electron microscopy (EM) studies and recently cryo-EM tomography (Cryo-ET) analysis revealed that excitatory PSD (ePSD) is mesh-like with a thickness of 20–50 nm, whereas the inhibitory PSD (iPSD) is sheet-like and ~12 nm thick [1–3]. PSD plays pivotal roles in synaptic plasticity, with changes in composition and morphology in response to different neuronal activities. Although the components of PSD have been largely identified, the assembly and regulation of PSD complexes are not well understood. ePSD is much better studied than iPSD, possibly due to its higher abundance and more distinctive structure. Here we focus on recent studies that shed light on how ePSD assemblies are organized. We discuss how these findings contribute to our understanding of ePSD functions in synaptic plasticity.

## The postsynaptic density

The PSD is a megadalton machinery that contains thousands of proteins, including transmembrane channels and receptors, adhesion molecules, scaffolding proteins, signalling enzymes, small GTPases and their regulators and actin cytoskeleton elements [1,4–6]. These proteins are present in a wide range of abundances, as demonstrated by proteomics analysis [7,8]. Scaffold proteins, including membrane-associated guanylate kinase (MAGUK), synapse-associated protein 90 (SAP90)/post-synaptic protein 95 (PSD-95)-associated protein (SAPAP), SRC homology 3 (SH3) and multiple ankyrin repeat domains protein (SHANK) and Homer families are present in high abundances, serving as a molecular platform onto which other functional proteins are recruited (Figure 1). EM coupled with immunostaining and superresolution imaging studies showed that the major scaffold proteins are arranged into layered organizations along the axo-dendritic axis [2,9–12]. By immuno-EM and antibody labelling, it was observed that PSD-95 is in a vertical orientation, with its N-terminus attached to the membrane via a palmitoylation group. The extended conformation of PSD-95 positions its N-terminal PDZ12 tandem close to the glutamate receptors at the membrane and the PDZ3-SH3-GK tandem to other scaffold proteins that lie towards the

**Figure 1**




Domain diagrams and interaction networks of key PSD components.
The diagram depicts the domain organization of five conserved PSD components (PSD-95, SynGAP, SAPAP, Shank and Homer) and their interactions with each other as well as with other PSD functional proteins (e.g. glutamate receptors and actin cytoskeleton). An arrow indicates the interaction between each pair of molecules, and a measured Kd value is given if available. For SAPAP, a phosphorylation-promoted interaction between its GK-binding repeats (GBRs) and the guanylate kinase (GK) domain of PSD-95 is shown. PSD-95, SynGAP, SAPAP, Shank and Homer are drawn in the same scale, and asterisks represent their C termini.

cytoplasmic side of PSD. SAPAP, Shank and Homer scaffolds may arrange into horizontal filaments that are in contact with the vertical filament of PSD-95. These scaffold proteins overall link adhesion molecules and receptors at the membrane to signalling proteins and actin cytoskeleton towards the inside of dendritic spines.

Recent developments in super resolution microscopy provide new insights into the lateral organization within the PSD. Scaffold proteins are not homogenously distributed in the PSD, but cluster into subsynaptic domains [11,13•,14•,15•,16•,17•,18,19•]. These nanodomains are ~80 nm in diameter and concentrate the

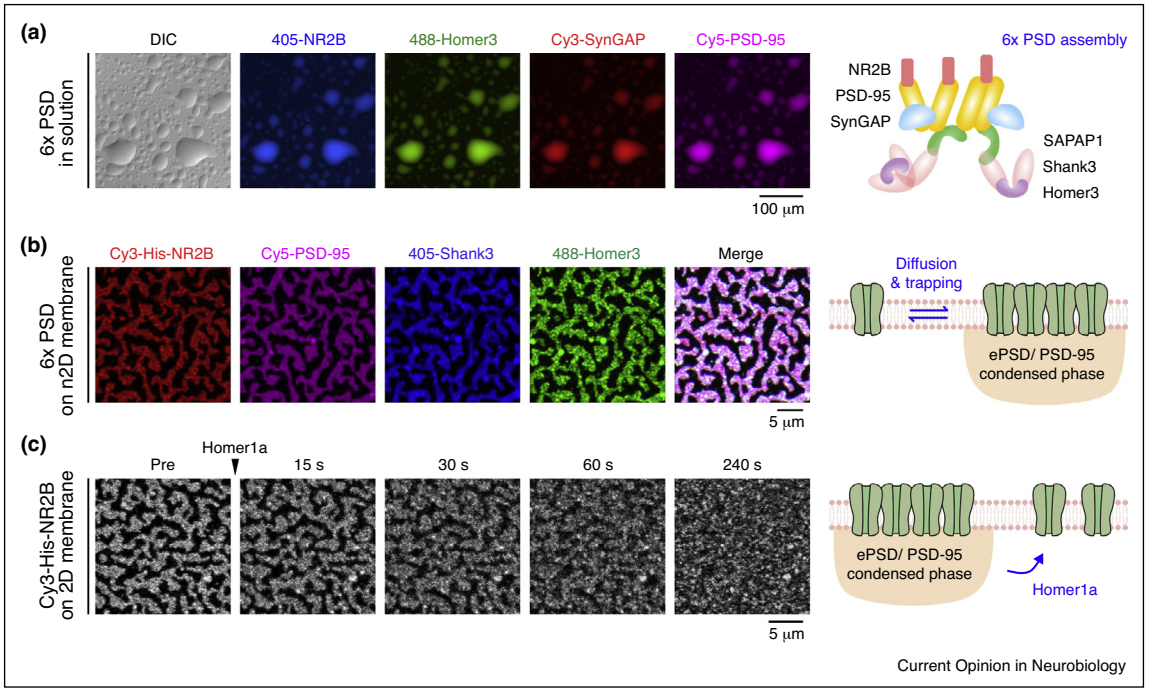
proteins by several folds when compared to their counterparts outside the clustering. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are also enriched in PSD-95-containing nanodomains, although not entirely co-localized [15•,17•]. About 20 receptors are detected in such clusters, significantly increasing the efficacy of synaptic transmission as suggested by simulation studies. Importantly, the expression level of PSD-95 determines the size and density of AMPA receptor nanodomains. Proteins within the subsynaptic clusters are less mobile than those outside the nanodomains, but they are still dynamic and can change over time or in response to different synaptic activities. Post-translational modifications also affect the assembly and disassembly of these nanodomains. It was shown that cycles of palmitoylation and depalmitoylation on PSD-95 affect the size and number of nanodomains [14•]. Furthermore, the nanoclusters of PSD-95 and AMPA receptors precisely align with presynaptic active zone proteins, forming trans-synaptic nanocolumns for efficient neurotransmission [20•,21•].

## PSD organization via phase separation

The traditional textbook view of organelle compartmentalization is that it is achieved via the presence of membrane bilayers. In this way, proteins are concentrated and enclosed within specific compartments. Some biomolecular complexes are, however, non-membrane bound. The PSD is one of such examples. The PSD is semi-membrane bound, with one side attached to the postsynaptic membrane and the other side exposed to the cytoplasm within the spine head [22]. The PSD complex is a self-assembling machinery and involves extensive protein interactions. Biochemical and biophysical studies have offered rich information on paired protein interactions [23]. However, it is still puzzling how the densely packed PSD proteins are stably maintained in the absence of a physical barrier. In addition, PSD components undergo dynamic changes in response to synaptic stimuli [24]. *In vitro* work using purified PSD proteins suggested that the phenomenon of liquid–liquid phase separation might underlie the organization of mega PSD assemblies [25••,26]. Synaptic GTPase-activating protein (SynGAP) is a GTPase activating protein that binds to PSD-95 via PDZ–PBM interaction [25••]. It is a negative regulator of synaptic strength due to downregulation of small G protein activities. Following LTP induction, SynGAP disperses from the PSD to release its inhibitory role [27,28]. Interestingly, recombinant PSD-95 and SynGAP proteins readily formed liquid condensates upon mixing, which phase separated from the bulk solution *in vitro* [25••]. Fluorescence recovery after photobleaching (FRAP) analysis showed that molecules within such condensates can freely exchange with the surrounding solution. PSD-95/SynGAP phase separation is promoted by trimerization of SynGAP. It was noted that disruption of its multimerization interface but not of binding to PSD-95 abolishes the ability of SynGAP to form phase condensates *in vitro*. In neurons, the monomeric mutant of SynGAP shows significantly reduced enrichment at the PSD under the basal state and elevated dispersion from the PSD upon LTP induction. Both *in vitro* and *in vivo* data prompt the hypothesis that the PSD might be organized via phase separation.

Inspired by this pioneering work, a recently published study using purified proteins provides exciting first steps towards reconstituting the PSD *in vitro* [29••]. Mixing the purified scaffold proteins including PSD-95, SAPAP1, Shank3 and Homer3 at physiological concentrations can form liquid-like condensates in solution (Figure 2a) and on 2D membrane bilayers (Figure 2b). These proteins are all multi-modular, capable of binding to each other and many other synaptic proteins and enzymes with high specificity and affinities (Figure 1) [23,30–35]. They can either form oligomers via self-multimerization or promote multimerization of their binding proteins by containing repeating protein binding domains/motifs (PDZ domains as an example) [36–39]. The multivalency and conformational flexibility of the PSD scaffold proteins and the large protein–protein interaction networks formed by these scaffold proteins are optimal for forming adaptable biological condensates via phase separation [40•,41•,42•,43•,44•]. It was noted that as complexity in the system increased by adding more components, the concentration threshold of liquid droplet formation was further lowered. Importantly, the *in vitro* condensates could cluster glutamate receptors (the C-terminal tail of NMDAR as presented in the study), enrich synaptic regulatory enzymes such as SynGAP and promote actin bundle formation via actin polymerization regulatory proteins such as cortactin (Figure 2a & b). The reconstitution system also showed layered organization features as observed *in vivo*. Removal of PSD-95 impacted on the phase separation ability of its direct binders such as SAPAP1 and glutamate receptors. Removal of Shank3, which is located more towards the cytoplasmic side of PSD [45,46], led to changes in the amount of Homer3 and SAPAP1 in the phase droplet but had much less influence on the upper layer proteins. The most dramatic effect was observed when SAPAP1 was absent in the reconstitution system. This may be explained by the linker role of SAPAP, which localizes to the middle layer of the PSD [47]. In contrast to scaffolding proteins, the removal of SynGAP, a regulatory enzyme, had little impact on the amount of droplet condensates, suggesting that SynGAP is a 'passenger' rather than a 'driver' of PSD phase separation [40•]. Importantly, the *in vitro* reconstituted PSD system is selective and can be modulated by regulatory mechanisms mimicking neuronal activities. Gephyrin is an abundant scaffolding protein in iPSD. It self-multimerizes and binds to dozens of proteins including GABA receptors, collybistin and actin-associated proteins [48]. Interestingly, Gephyrin is excluded

**Figure 2**




Reconstituted 6× PSD in solution and on supported lipid membrane bilayers.
**(a)** DIC and fluorescence imaging showing that 6× PSD formed phase condensates in solution, with PSD-95, Shank3, SynGAP and NR2B tail highly enriched in spherical droplets. Note that SAPAP1 and Homer3 were unlabelled and thus invisible. Schematic diagram on the right showing the principle of 6× PSD assembly.
**(b)** 6× PSD underwent phase separation on supported lipid membrane bilayers. Freely diffused NR2B tails were largely clustered by 6× PSD phase condensates, mimicking the dynamic diffusion and synaptic trapping behaviors of glutamate receptors in living neurons.
**(c)** Homer1a can dissolve 6× PSD condensates, and pre-clustered NR2B tails disassembled after addition of purified Homer1a protein.
Figure adapted from [29••].

from the droplet condensates formed by ePSD scaffolds, although the proteins are homogenously mixed in the dilute phase. The observation of mutual exclusion of inhibitory and excitatory scaffold proteins may have important implications in understanding why excitatory and inhibitory synapses are never found to co-exist physically even if a single dendritic spine is dually innervated by both excitatory and inhibitory presynaptic cells [49]. Homer1a is a short, monomeric splice variant of Homer1 protein, and acts as a dominant negative effector of synaptic functions [50]. Strikingly, overexpression of Homer1a in the reconstitution system dispersed the condensed phase of PSD proteins due to reduced complexity of the PSD network (Figure 2c). This observation correlates with global downscaling of the PSD size induced by sleep when Homer1a expression level is highly upregulated [51,52].

## Functional implications of phase separation driven PSD organization

The physiochemical properties of phase separation-driven condensates facilitate several key functional features of postsynaptic scaffold proteins, as well as their binding proteins and enzymes, in synapse development and synaptic plasticity. Each PSD is formed autonomously, and this formation is driven by phase separation when protein concentrations reach certain thresholds. Such threshold concentration-dependent PSD formation has huge implications on the total amount of proteins that needs to be synthesized in each neuron as well as on the mechanisms of protein trafficking to synaptic terminals. Threshold concentration-dependence may also offer a possible explanation for the synaptic functional alterations that result from mutations or loss of one allele of synaptic encoding genes found in patients with brain disorders. The PSD has no repeating pattern in terms of its morphology and molecular composition (some spines/PSDs are large, and others are relatively small; each PSD differs in the amount of individual constituents), and such distinct and heterogeneous pattern can be easily accomplished by changes in the amount of proteins in each synapse. It has long been unclear how PSD components are stably maintained against Brownian motion, even though there is a steep protein

concentration gradient from the PSD to the cytoplasmic pool. Phase separation allows molecules to stay in a condensed state, meanwhile allowing them to freely exchange with their counterparts in the dilute cytoplasm. In addition, the condensed phase facilitates biochemical reactions by enriching enzymes or substrates or by simply concentrating building blocks, as is the case in centrosome assembly [53]. Phase separation can also sequester biomolecules to stop or postpone reactions, such as in stress granules [54]. The PSD itself is not uniform, as it has a layered organization along the axo-dendritic axis (revealed by EM studies) and distinct nano-clustering enriched with scaffolding proteins and glutamate receptors (shown by superresolution studies). The layered organization and the subsynaptic domains of the PSD may indicate two different types of subsegregation in phase separation models that have been recently reported: phase-to-phase and phase in phase [55–58]. In general biological concepts, such subsegregation of materials in living cell can only be truly achieved by the presence of multi lipid bilayers like in mitochondria or chloroplasts. By phase separation, however, one may get several subphases, each with different functions, in one aqueous mixture without the help of membrane segregation. Although the observation and biological implications of more delicate subsegregation in a single condensed phase are clear, the detailed molecular mechanisms behind this are still largely unknown.

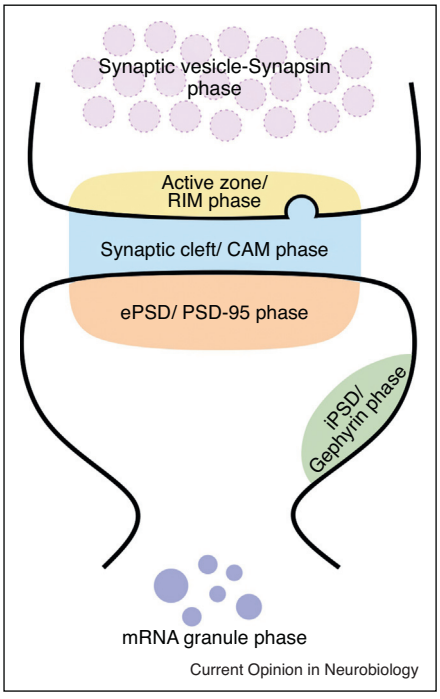
In early LTP, molecules with positive functions (like AMPA receptor/AMPA receptor regulatory protein (TARP), calcium/calmodulin-dependent kinase II (CaMKII), actin) undergo extremely fast translocation to the PSD, while 'brakes' like SynGAP are rapidly dispersed to the dendritic shaft. This fast molecular rearrangement is hard to accomplish if the entire PSD assembly is enclosed by lipid membranes, since the time-consuming process of cargo sorting and delivery by vesicles is not favored in such a short time window. This problem can be perfectly solved if the whole PSD is organized through phase separation, which allows rapid exchange of molecules inside and outside of the condensed phase. Post translational modifications may be one of the potential mechanisms underlying how a given synaptic protein could change its synaptic localization in response to different neuronal activities, as several types of protein modifications have been reported to monitor the phase separation properties in non-PSD systems [43•,58–62]. CaMKII is one of the most abundant synaptic kinases in the PSD, and its kinase activity is specifically upregulated during LTP, possibly by modifying numerous PSD targets like transmembrane TARP, GluN2B, SynGAP, SAPAP and subsequently change their behaviors [63–66]. Beside protein phosphorylation, PSD phase separation properties could also be regulated by other modifications including protein glycosylation, lipidation, methylation and so on. These modifications on synaptic proteins may largely affect their phase separation efficiency by directly modulating their molecular features and protein–protein interactions.

## Conclusions and perspective

Since the first observation of dense thickenings beneath the postsynaptic membranes of excitatory synapses, great efforts have been made to understand how such highly enriched PSD assemblies not enclosed by membranes can autonomously form, stably exist, and at the same time dynamically change their constitutes in response to synaptic stimuli. Systematically investigating the molecular mechanisms governing the post-synaptic compartmentalized assemblies in living neurons has been difficult, partly due to the small size of these structures, which makes them unsuitable for many optical imaging methods, but also the non-repeating structural features of synapses (e.g. too irregular to derive atomic scale structures by existing biophysical methods). *In vitro* studies by conventional biochemical and biophysical methods using isolated proteins in pairs are important intermediate steps towards understanding the condensed synaptic assemblies [23], but such approaches are unlikely to be able to faithfully recapitulate the protein interaction networks in the condensed postsynaptic compartments. The phenomenon of phase separation-driven PSD assembly has changed our textbook view of synaptic organization (Figure 3).

However, many questions remain to be answered. For instance, how does ePSD specifically and selectively repel iPSD proteins? How do subsynaptic nanodomains assemble within the highly condensed PSD? Does the size of phase condensates at the postsynapse help define the boundary and hence the alignment with presynaptic termini or vice versa? What unique features, in terms of synaptic signalling, do condensed ePSD molecular assemblies have with respect to conventional protein–protein interaction networks in dilute solution? Might one be able to interpret the impact of mutations in genes encoding synaptic proteins on synapse formation and function with new angles if taking the condensed phase assembly of PSD into account? Will the concept of phase separation-mediated formation of PSD offer a new opportunity to understand psychiatric disorders caused by mutations of genes encoding synaptic proteins and consequently offer new avenues for developing therapeutic methods for these diseases? The *in vitro* reconstitution system presented in recent studies provides a new platform to investigate these questions. The current system comprises mainly scaffolding proteins, and it will be of great interest to analyse the enrichment of many other PSD proteins into the condensed phase. Meanwhile, the system needs to be further developed for more quantitative descriptions. For example, single molecule tracking techniques may be combined with the 2D membrane system for future membrane protein or receptor studies. Theoretical work should be conducted in order to understand the underlying physical and

**Figure 3**




Formation of other subsynaptic compartments may also be mediated by phase separation.
Similar to ePSD, presynaptic active zone and synaptic cleft also form condensed structures observed by EM. Phase separation of RIM/RIMBP-containing presynaptic scaffold proteins and multimeric cell adhesion molecules (CAM) may be the underlying mechanism for the formation of these structures, respectively. Presynaptic protein synapsin can form a condensed liquid phase that captures synaptic vesicles [67]. mRNA-containing granules are observed on dendritic shafts and may be related to the local synthesis of synaptic components [68]. How to target proteins and mRNAs to specific subsynaptic compartments/phases? How do distinct compartments/phases communicate with each other? How to prevent coalescence among different membraneless compartments/phases in a tiny dendritic spine? Understanding these questions will be pivotal for understanding synapse formation, regulation and plasticity.

chemical properties of the protein condensates. The system might be modulated via bioengineering/chemical biology tools to precisely control phase separations for mimicking the range of neuronal activities. Of course, one of the most pressing directions for future research is to establish whether phase separation-mediated PSD condensate formation and dynamic regulation is indeed in operation in live neurons in animal brains.

## Conflict of interest statement

Nothing declared.

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