

Phase separation at the synapse

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Emerging evidence indicates that liquid–liquid phase separation, the formation of a condensed molecular assembly within another diluted aqueous solution, is a means for cells to organize highly condensed biological assemblies (also known as biological condensates or membraneless compartments) with very broad functions and regulatory properties in different subcellular regions. Molecular machineries dictating synaptic transmissions in both presynaptic boutons and postsynaptic densities of neuronal synapses may be such biological condensates. Here we review recent developments showing how phase separation can build dense synaptic molecular clusters, highlight unique features of such condensed clusters in the context of synaptic development and signaling, discuss how aberrant phase-separation-mediated synaptic assembly formation may contribute to dysfunctional signaling in psychiatric disorders, and present some challenges and opportunities of phase separation in synaptic biology.

Eukaryotic cells orchestrate numerous biochemical reactions spatiotemporally by segregating each cell into structurally and functionally distinct compartments. In addition to classical membrane-enclosed cellular compartments, increasing recent evidence reveals a diverse class of cellular compartments that either lack membranes or are not enclosed by membranes, formed by a physical process known as liquid–liquid phase separation and frequently referred to as membraneless compartments or biological condensates (Fig. 1 and Box 1).

Observation of membraneless condensates may date back to more than 100 years ago when Ramon y Cajal observed dense spots in the nuclei of silver-stained neurons¹. These are now known as Cajal bodies, mRNA processing machineries localized within the nucleus and enriched in proteins and RNAs. Some well-recognized examples of membraneless condensates include various nuclear bodies, P granules, stress granules and processing bodies^{2–5}.

Neurons take cellular compartmentalization to extremes due to their elaborate morphologies and high degree of polarity. In addition to membrane-enclosed organelles and membraneless condensates common to other cell types, neurons contain a unique type of membrane-semi-enclosed compartments known as synapses, which are molecular apparatuses dictating signal processing and transmissions in all nervous systems (Fig. 2). Neither pre- nor postsynaptic compartments are enclosed by membrane bilayers. Underneath the postsynaptic plasma membranes of each synapse is a condensed protein-rich sub-compartment known as the postsynaptic density (PSD), a structure responsible for receiving, amplifying and storing signals initiated by presynaptic cells. PSDs are composed of densely packed proteins forming mega-assemblies a few hundred nanometers in width and ~30–50 nm thick^{6–8} (Fig. 2b). In the presynaptic compartments, a layer of electron-dense material beneath the plasma membranes, known as the active zone, can also be observed by electron microscope⁹, and these electron-dense materials are also composed of densely packed proteins¹⁰. Active zones play critical roles in docking and priming readily releasable synaptic vesicles (SVs) and in the clustering and positioning of voltage-gated Ca²⁺ channels (VGCCs) at subregions of the presynaptic active zone membrane to regulate the speed and strength of neurotransmitter releases^{10,11}. Additionally, the reserve pools of SVs, which account for up to 90% of total vesicles^{12,13}, are clustered together with

proteins such as Synapsin and Intersectin and situated distal to the readily releasable pool of SVs docked to active zones (Fig. 2b).

A series of recent studies have provided evidence that formations of the condensed PSDs, presynaptic active zones and the clustered reserve pool SVs may involve phase-separation-mediated molecular assemblies^{14–17}. Here we review these recent findings. We try to align the concepts and findings of phase separations in synapses with years of research in synaptic signaling complex trafficking, organization and clustering; and we try to provide implications of phase separation on synaptic formation and plasticity, as well as on psychiatric disorders. This review will not cover phase-separation-mediated formation of pathological aggregates that may cause various neurodegenerative diseases. Readers are referred to several reviews on this topic^{18–20}.

Formation of PSD assembly via phase separation

PSDs are composed of densely packed protein mixtures without membrane enclosures. When initially discovered ~60 years ago^{21,22}, PSDs were observed as electron-dense thickenings beneath postsynaptic plasma membranes and thus open to the cytoplasm of dendritic spines. Subsequent biochemical purification of PSDs revealed that the vast majority of molecular components of isolated PSDs are proteins²³. Extensive biochemistry and electron microscopy (EM) investigations in the past 60 years, together with more recent proteomic studies, have elucidated that PSDs are composed of hundreds of different proteins with a wide range of abundances^{24–29}, and these proteins form interconnected disc-shaped molecular assemblies^{6,7,30}. Scaffold proteins such as PSD-95³¹, GKAP (also called SAPAP)³², Shank3³³ and Homer³⁴ are major components of PSDs. A prominent feature of PSDs is that the dense assembly is attached on one side to the postsynaptic plasma membranes and on the other side is exposed to the dendritic spine cytoplasm. Thus, PSDs are a type of condensed subcellular compartments that are not enclosed by lipid membranes.

PSDs are dynamic molecular assemblies. Dendritic spines of excitatory synapses are highly dynamic both during development and in adult animals, and there are good correlations between the volume of a dendritic spine and its synaptic strength^{35,36}. EM studies revealed that the volume of a dendritic spine is nearly linearly correlated with its PSD area and with the number of vesicles in the

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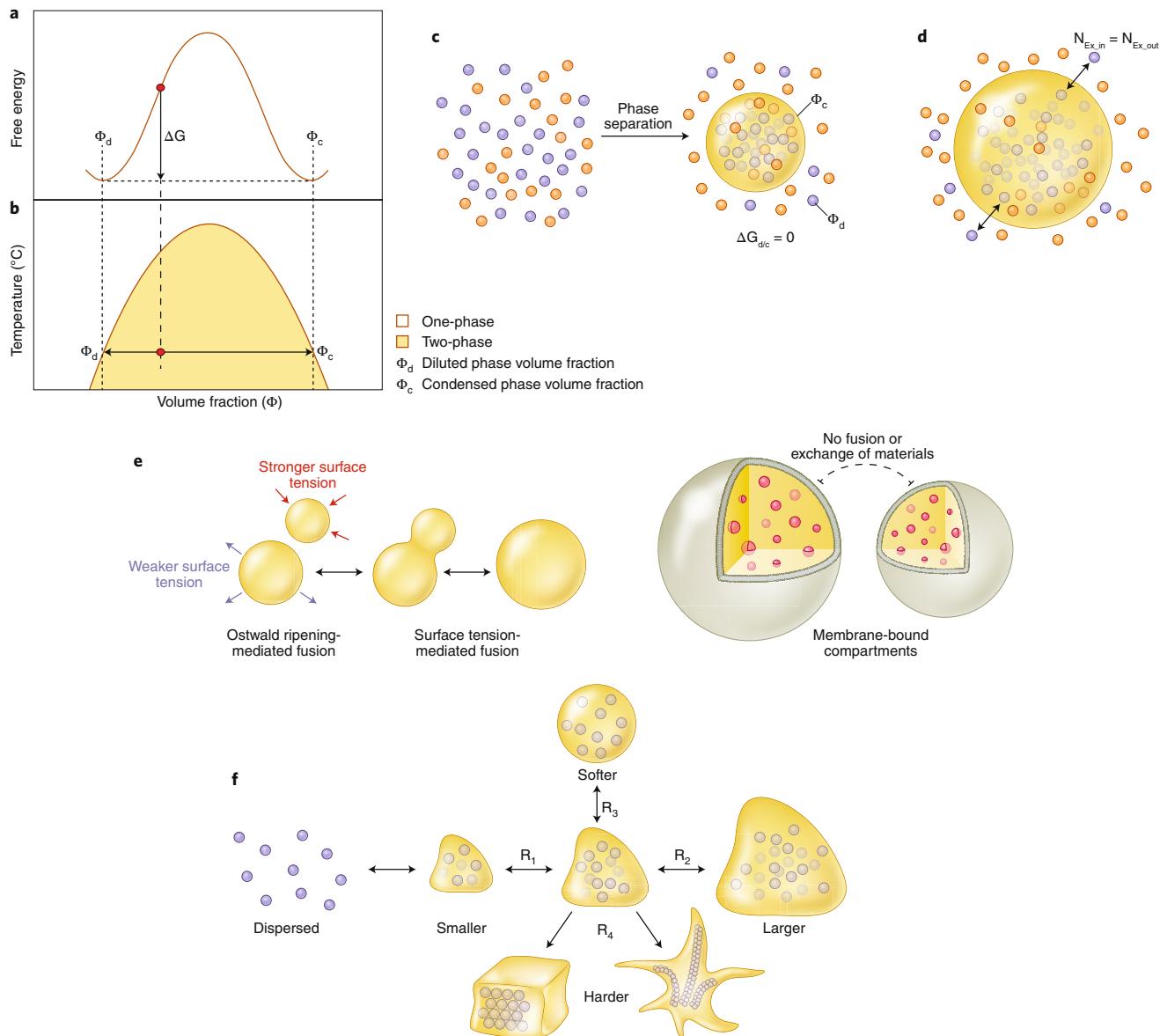


Fig. 1 | Basic principles of phase separation illustrated by a simple two-component system. **a**, Free energy diagram showing phase separation of a two-component system (for example, a protein indicated by blue dots in aqueous solution indicated by golden dots in **c**) under one condition. A uniformly mixed system can undergo phase separation by lowering the free energy (ΔG) to its minimal level, resulting in a two-phase system: a dilute phase with a lower protein concentration (Φ_d , expressed as the fraction volume for the dilute phase) and a condensed phase with a higher protein concentration (Φ_c , the fraction volume for the condensed phase). **b**, Phase diagram of the two-component system constructed by plotting the free energy minima as the function of temperature. The curve indicates a sharp boundary (or the threshold concentration) of the system undergoing from a homogenous single-phase state to a two-phase state. **c**, Phase separation results in formation of a condensed phase enriched with a particular molecular component (here represented as blue dots) and surrounded by the diluted phase with a much lower concentration of the blue dot molecules. **d**, After phase separation, the components between the two phases can freely exchange. However, there is no net flow of components between the two phases, as the number of molecules (N) exchanged in and out are equal. **e**, Left: the condensed phase droplets can fuse with each other into larger droplets, as such fusion can reduce the surface tensions of smaller droplets. Additionally, small droplets can spontaneously shrink, and released molecules can be absorbed by other, larger droplet sizes, a process known as Ostwald ripening. Right: for membrane-separated compartments, spontaneous compartment fusion or materials exchange do not occur. **f**, Biological condensates are highly plastic and can undergo different forms of changes with regulatory inputs (R). A droplet size can be made to shrink or enlarge by simply decreasing or increasing the amount of materials in the system (R_1 and R_2). A droplet can become softer or harder upon different regulatory modifications (R_3 and R_4 ; for example, formation of reversible gel-like protein aggregates or even some non-reversible solid-like protein deposits).

presynaptic bouton³⁷. Enlargement or shrinkage of PSDs involves addition or removal of new proteins such as glutamate receptors and their downstream scaffolding proteins from the PSD assembly^{35,38}. Additionally, AMPA receptors within and outside PSD are constantly undergoing exchange, and receptors within the condensed PSD are still mobile but with much slower diffusion rates^{39–42}.

Scaffold proteins such as PSD-95, GKAP, Shank3 and Homer in synapses also undergo dynamic movements, forming distinct condensed nanoclusters^{42–46}. Therefore, the PSD assembly has key features of membraneless biological condensates (Box 1): proteins in PSDs are highly concentrated; PSD condensates can grow or shrink; components within the condensed PSDs are mobile and can

Box 1 | Phase-separation-mediated formation of membraneless compartments

When biomolecules are dissolved in water, these molecules tend to mix homogeneously with water due to the mixture's tendency to increase its entropy (Fig. 1c). However, if biomolecules can interact with each other, then they may start to demix from water, forming two distinct phases via phase separation (Fig. 1c). In this two-phase system, there is no free energy difference between the two phases. The diffusion chemical potential (μ) generated by the concentration gradient of biomolecules between the two phases is offset by the net free energy gain ($\Delta\Delta G$) of increased binding of biomolecules in the condensed phase, so the system is at a thermodynamic equilibrium. Nonetheless, molecules in the condensed phase are constantly exchanging with those in dilute phase, albeit that the numbers of molecules exchanging in and out at any given time are the same (i.e., the system is at a dynamic equilibrium; Fig. 1d). The free energy diagram of a two-phase mixture at a specific condition, illustrated in Fig. 1a, dictates that the system can spontaneously reach to two local minima, corresponding to ϕ_d and ϕ_c . The phase diagram in Fig. 1b indicates that if the concentration of biomolecules is between those corresponding to ϕ_d and ϕ_c , the mixture will spontaneously demix, forming a condensed phase and a dilute phase with concentrations corresponding to ϕ_c and ϕ_d , respectively. Additionally, during the phase separation process, large droplets will continue to grow larger at the expense of shrinkage of small droplets, a phenomenon known as Ostwald ripening¹⁰⁹. When two droplets meet, they will fuse due to the lower surface tension of the larger droplet (Fig. 1e, left). In a membrane-delimited compartment, molecules within and outside the compartment cannot freely exchange (Fig. 1e, right).

Condensed assemblies formed via phase separation in cells can almost always be viewed as active condensates, meaning that the condensed phases are subject to various modulations (Fig. 1f). For example, phase-separated condensates can become smaller or even completely dissolve on receiving certain stimuli. The condensed phase can also become larger if new materials are added or existing components are modified. The material properties of the condensed assembly can also be modified to become softer or harder in response to various conditions^{110,111}. In extreme cases, the formed condensates can form irreversible solids under pathological conditions (for example, amyloid fibers in Alzheimer's patients).

exchange with corresponding molecules in the dilute cytoplasm of dendritic spines.

PSD may form via phase separation: initial observation. The first hint suggesting that the PSD may form via phase separation came from a recent study of the interaction between PSD-95 and SynGAP¹⁴. SynGAP is a negative regulator of synaptic strength by stimulating the GAP activities of small G proteins such as Ras and Rap^{47–52}. SynGAP mutations are associated with epilepsy, intellectual disability and autism^{51,53}. SynGAP exists at a very high abundance with a near stoichiometric ratio to PSD-95 in PSD²⁴. The interaction between SynGAP and PSD-95 is highly specific and stable¹⁴. Apparently, synaptic activities are highly sensitive to the dosage of SynGAP, as SynGAP haploinsufficiency is highly penetrant in causing intellectual disability and autism⁵⁴. Thus, in addition to functioning as a GTPase activating enzyme, SynGAP may play certain nonenzymatic roles in PSD via specifically binding to PSD-95.

PSD-95 and SynGAP, either as purified proteins mixed in test tubes or as proteins co-expressed in heterologous cells, formed

spherical droplets via phase separation¹⁴. Phase separation of the PSD-95–SynGAP complex requires homotrimer formation of SynGAP as well as SynGAP-binding-induced PSD-95 multimerization^{14,55,56}, showing that the PSD-95–SynGAP complex phase separation is governed by the specific and multivalent interaction between the two proteins⁵⁷. A SynGAP mutant, which retained the same binding affinity to PSD-95 but was a monomer, was completely incapable of undergoing phase separation with PSD-95¹⁴. It should be noted that the PSD-95 and SynGAP used in the study by Zeng et al.¹⁴ were not full-length proteins. Including the first two PDZ domains in PSD-95 further increases interaction valency between PSD-95 and SynGAP⁵⁸ and thus should enhance phase separation of the complex⁵⁷. Similarly, a large segment of intrinsically disordered region between the catalytic domain and the coiled coil domain of SynGAP may also modulate phase separation of the PSD-95–SynGAP complex^{2,3}.

Hippocampal neurons with wild-type SynGAP replaced by the monomeric SynGAP mutant showed defective PSD targeting of SynGAP, presumably due to the impaired co-enrichment with PSD-95 via phase separation. Neurons expressing this monomeric SynGAP mutant were hypersensitive to weaker stimulations¹⁴, which provides a possible mechanistic explanation for SynGAP mutation-induced hyperexcitation in patient brains⁵⁴. Phase-separation-mediated autonomous co-condensation of PSD-95 and SynGAP led the authors to suggest that highly condensed PSD assemblies observed under EM may form via phase separation¹⁴.

PSD formation via phase separation: a biochemical reconstitution approach. Based on the initial observation of the PSD-95–SynGAP complex phase separation, Zeng et al. tried to reconstitute the PSD assembly first by using four purified PSD scaffolding proteins: PSD-95, GKAP, Shank and Homer¹⁵. These four scaffold proteins serve to connect the transmembrane ion channels and receptors on the postsynaptic plasma membranes to the actin cytoskeleton at the interface between PSD and spine cytoplasm^{59–61} by forming a large protein network via highly specific and strong interactions (Fig. 3a). To obtain high quality recombinant proteins, the authors used fragments of GKAP and Shank instead of the full-length proteins for the reconstitution experiments (Fig. 3a). When mixed at a 1:1:1:1 ratio, the mixture underwent phase separation, forming spherical droplets with all four proteins co-condensed (Fig. 3b). Importantly, the phase separation of the four-scaffold-protein mixture occurred at individual protein concentrations as low as 1 μ M, which is well below the concentrations of these proteins in synapses^{28,62,63}. The specific and multivalent interactions connecting the PSD scaffold proteins into a large protein network is critical for the phase separation and co-condensation of the four-protein PSD mixture. A GKAP mutant with its PDZ-binding motif (PBM) removed was still able to form phase separation with PSD-95, and a Shank3 and Homer3 mixture could also form condensed droplets via phase separation. However, the PSD-95–GKAPΔPBM phase was found to demix from the Shank3–Homer3 phase (Fig. 3b). The condensates formed by the four scaffold proteins can recruit and co-condense the NR2B tail and SynGAP (Fig. 3c). Thus, PSD scaffold condensates appear to be able to cluster receptors and concentrate enzymes, two hallmark features of scaffold proteins in synapses^{64,65}.

Since PSDs are formed right beneath the synaptic plasma membranes, the reconstitution of PSD assemblies was also tested by tethering the NR2B tail to supported lipid bilayers (SLB) and then adding the synaptic scaffold proteins and SynGAP to the SLB¹⁵. Interestingly, the reconstituted PSD assembly on the SLB also underwent phase separation via spinodal decomposition (Fig. 3d). The concentration threshold for the PSD protein mixture to form phase separation on a two-dimensional SLB is lower than in a three-dimensional solution. The formed PSD condensates have a web-like structure, reminiscent of perforated PSDs observed by EM.

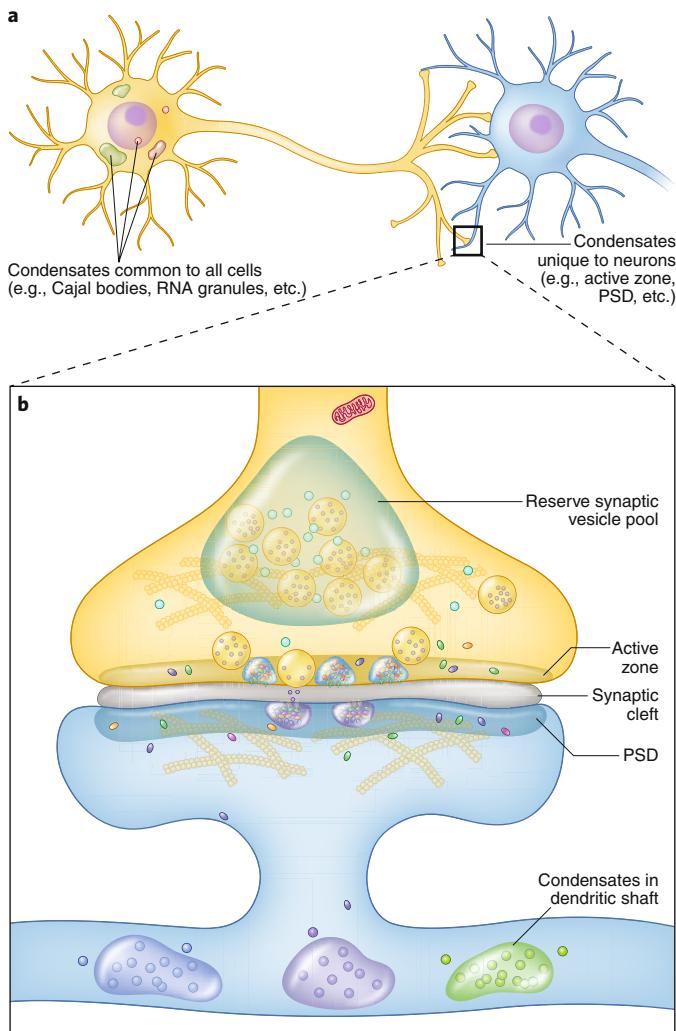


Fig. 2 | Phase separation in neurons. **a**, Schematic diagram showing two highly polarized neurons communicating with each other via synapses. Neurons also contain various biological condensates common to other cell types, including RNA granules and stress granules in soma, as well as Cajal bodies and nucleolus in nuclei. Additionally, neurons contain unique biological condensates such as PSDs and active zones specifically found in synapses. **b**, Overview of various biological condensates in synapses. In a presynaptic bouton, the reserved pool of SVs may be clustered by proteins such as Synapsin via phase separation. Formation of presynaptic active zones and PSD assemblies may also involve phase separation. It is possible that the synaptic cleft may also contain condensed protein assemblies. Additionally, dendritic shafts may contain various biological condensates, such as RNA granules, for local protein synthesis.

Both the NR2B tail and SynGAP (or the truncated SynGAP used in the study) mainly act as ‘clients’ of the condensed PSD assembly, as dropouts of either of the two proteins did not obviously change the phase separation of the four-scaffold-protein mixtures.

Using this reconstituted PSD system combined with electrophysiology studies, it has been demonstrated that AMPA-receptor regulatory proteins (TARPs) can be clustered into the PSD condensates via phase separation⁶⁶. The clustering of TARPs into the PSD condensates is mediated via a highly specific and multivalent interaction between the entire C-terminal tail of each TARP and the PDZ12 tandem of PSD-95. Such multivalent TARP-PSD-95 interaction is essential for AMPA-receptor synaptic transmission in mice hippocampal neurons⁶⁶. This study provides an important piece of

evidence supporting the concept that phase-separation-mediated PSD assembly formation and regulation are linked with physiological functions of synapses.

Phase separation in presynaptic boutons

The reserve pool and readily releasable pool SVs are organized by distinct proteins and can exchange with each other^{6,13,67} (Fig. 4a). Synapsins are very abundant in presynaptic boutons and essential for clustering the bulk reserve pool of SVs^{68–70}. Active zone proteins, such as RIMs and RIM-BPs, are critical for anchoring readily releasable pool SVs to fusion sites¹⁰. Perturbations of synapsins dramatically diminished the reserve pool SVs, but the readily releasable pool SVs remained intact^{68,69,71}. Correspondingly, genetic removal of key active zone proteins, including RIMs, RIM-BPs and ELKS, disrupted active zone formation and eliminated readily releasable pool SVs^{72,73}. The mechanisms governing the organization and clustering of these two pools of SVs have remained unclear until recent studies indicating that these two pools of SVs may be clustered via phase-separation-mediated formation of protein-SV condensates^{16,17}.

Phase-separation-mediated clustering of the reserved pool of SVs. Synapsins are known to interact with SVs for decades^{74,75}. Synapsin I, encoded by *SYN1*, is most abundant in CNS⁷⁶. The N-terminal portion of Synapsin I (amino acids (aa) 110–420) contains a dimeric ATPase synthase-like domain^{77,78}, and the C-terminal half of the protein is intrinsically disordered. The N terminus of Synapsin I is known to directly bind to phospholipids, and thus may link the protein with SVs^{79,80}. Synapsin I contains several characteristic features for forming condensed assembly via phase separation^{2,3}. Indeed, Milovanovic et al. showed that purified full-length Synapsin I can undergo phase separation¹⁶. The C-terminal intrinsically disordered sequence of Synapsin I was found to be responsible for this, and an intersectin fragment containing five SH3 domains can promote phase separation of Synapsin I¹⁶, likely via increased network complexity or valency of the Synapsin–Intersectin mixture⁵⁷. Strikingly, the condensed phase formed by Synapsin I can cluster SV-mimicking liposomes containing negatively charged lipids.

Synapsins were identified as presynaptic phosphoproteins⁸¹. Sustained synaptic stimulations trigger further phosphorylation of Synapsin I and result in its dissociation from SVs^{79,82}. CaMKII is known to bind to and phosphorylate Synapsin in the intrinsically disordered region⁸³. Interestingly, Ca²⁺ CaM-bound CaMKII can be recruited to the Synapsin I condensates. Initiation of CaMKII-mediated phosphorylation by adding a low concentration of ATP into the phase-separated mixture dispersed both Synapsin I condensates and the Synapsin I-liposome co-condensates¹⁶, indicating that CaMKII phosphorylation-induced Synapsin I dissociation from SVs may be a result of Synapsin dispersion from the Synapsin-SV condensates (Fig. 4b).

Phase-separation-mediated active zone complex formation.

Analogous to PSDs, presynaptic active zones are also structurally characterized by a layer of electron-dense materials beneath the presynaptic plasma membranes⁹. Decades of efforts using genetic, cell biology and electrophysiology studies have uncovered the molecular composition of the dense active zone assembly. A set of scaffold proteins, including RIM, RIM-BP, ELKS, Munc13, Liprin and CASK, are major components of active zones, and these proteins are conserved from invertebrates to vertebrates^{10,84}. A recent super-resolution microscopic imaging study showed that RIM forms nanocluster distributions within the dense active zones, instead of a randomly diffused distribution⁸⁵, in line with the disappearance of the dense projection structures of active zones observed in RIM–RIM-BP or RIM–ELKS double-knockout mice^{72,73}. Voltage-gated Ca²⁺ channels are clustered at subregions on the active zone membranes and positioned near the SV fusion

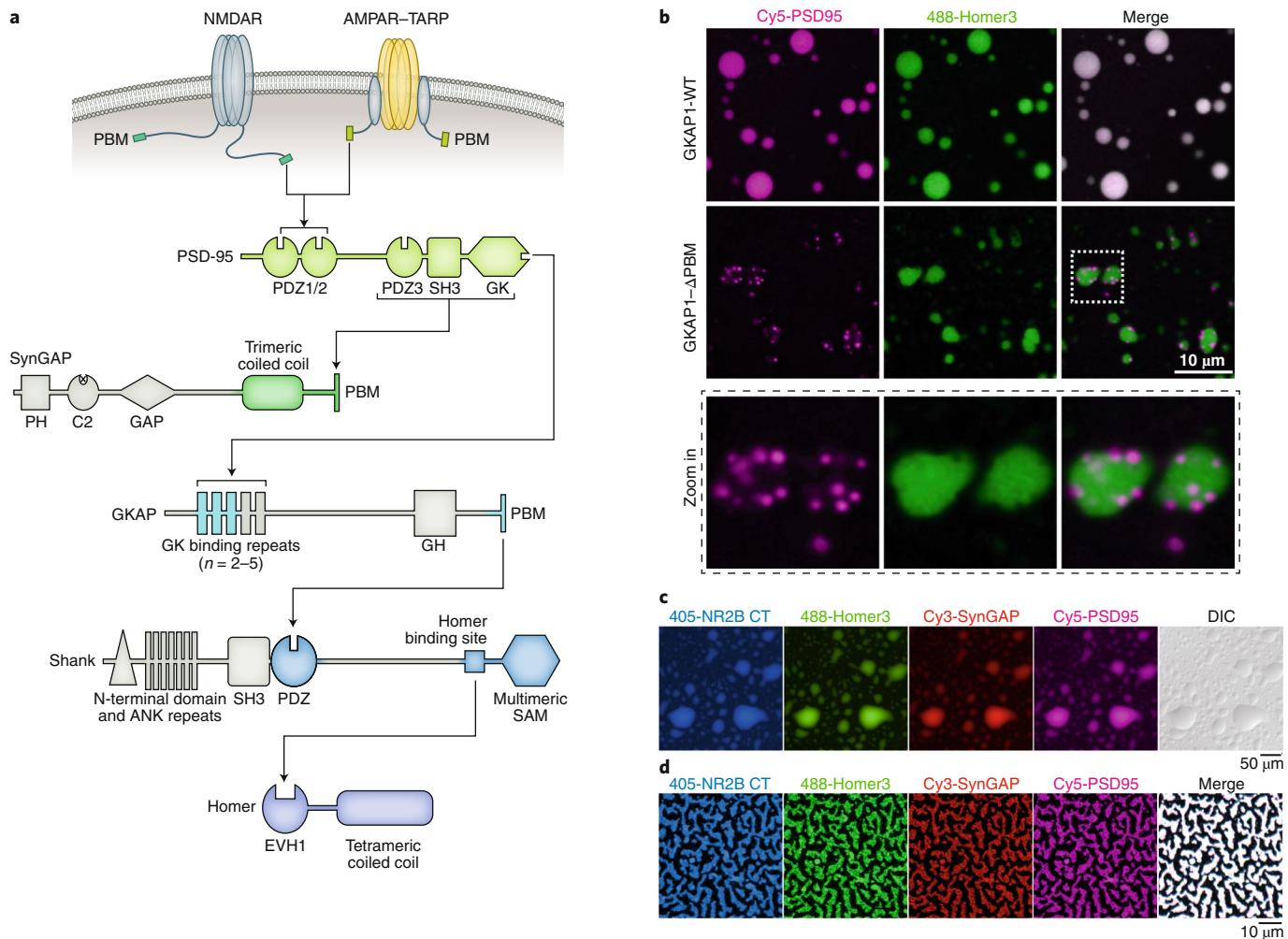


Fig. 3 | Phase-separation-mediated formation of PSD assemblies. **a**, Schematic diagram showing the domain organizations and interaction networks of key PSD components. These PSD components contain either repeating domains or motifs for protein–protein interaction or self-oligomerization domains to increase network valency. The PSD network is formed by multivalent, specific, strong protein–protein interactions and can undergo phase separation at the physiological protein concentrations in vitro. In addition to PSD-95 and Homer, fragments of SynGAP, GKAP and Shank (highlighted with colors) were used in the reconstitution experiments¹⁵ as described in **b–d** below. **b**, Confocal images showing that GKAP, the linker between the NR2B-PSD-95 layer and the Shank-Homer layer, is essential for forming an integrated PSD network assembly in vitro via phase separation. In this scheme, components consisting of NR2B-CT, PSD-95 (Cy5-labeled), GKAP, Shank3 and Homer3 (Alexa Fluor 488-labeled) underwent phase separation, with labeled PSD-95 and Homer shown to be perfectly co-localized. Disrupting the specific interaction between GKAP and Shank3 by removing the C-terminal last 15-residue PBM of GKAP showed that the resultant NR2B-CT-PSD-95-GKAP1-ΔPBM phase (purple) is de-mixed from the Shank3-Homer3 phase (green). **c**, Fluorescence and differential interference contrast (DIC) images showing the phase separation of the PSD scaffolding proteins mixture composed of PSD-95, GKAP, Shank3 and Homer3 in solution. Such PSD scaffold condensates could further cluster NR2B and enrich SynGAP. GKAP and Shank3 were not labeled and are thus not visible. **d**, Confocal images showing the clustering of membrane-tethered NR2B by PSD-95, SynGAP, GKAP, Shank3 and Homer3 on the SLB. GKAP and Shank3 were not labeled and are not visible. Images adapted from ref. ¹⁵, Cell Press.

sites to regulate both the speed and strength of neurotransmitter releases induced by action potentials^{10,11,85–87}. Like the PSD assemblies, presynaptic active zones appear to be self-assembled and condensed protein network structures right beneath but not enclosed by plasma membranes (Fig. 4c).

Using an in vitro reconstitution approach, Wu et al. recently demonstrated that the purified RIM and RIM-BP mixture undergoes phase separation under physiological protein concentrations¹⁷. Multiple proline-rich sequences distributed along the entire length of RIM can specifically bind to the three SH3 domains of RIM-BP, and such multivalent interactions drive the phase separation of the complex. Additionally, several intrinsically disordered sequences in RIM further promote the phase separation of the RIM-RIM-BP complex. It is speculated that combinations of specific multivalent

bindings, together with weak and promiscuous interactions mediated by intrinsically disordered sequences, are advantageous for forming biological condensates with high specificity and defined biological functions (for example, the active zones).

Fast and accurate neurotransmitter release critically depends on both the density of clustered VGCC on presynaptic plasma membranes and on the proximity of the clustered VGCC to calcium sensors at the SV fusion sites^{88,89}. RIM and RIM-BP are known to be essential for localizing and clustering VGCC at active zones, as well as for coupling of clustered VGCC to release sites^{90,91}. Fitting with these functional observations, the cytosolic tail of VGCC can be recruited to the RIM-RIM-BP condensates via direct bindings of the Ca^{2+} channel tail to both RIM and RIM-BP. The Ca^{2+} channel tail in return can further promote phase separation of the

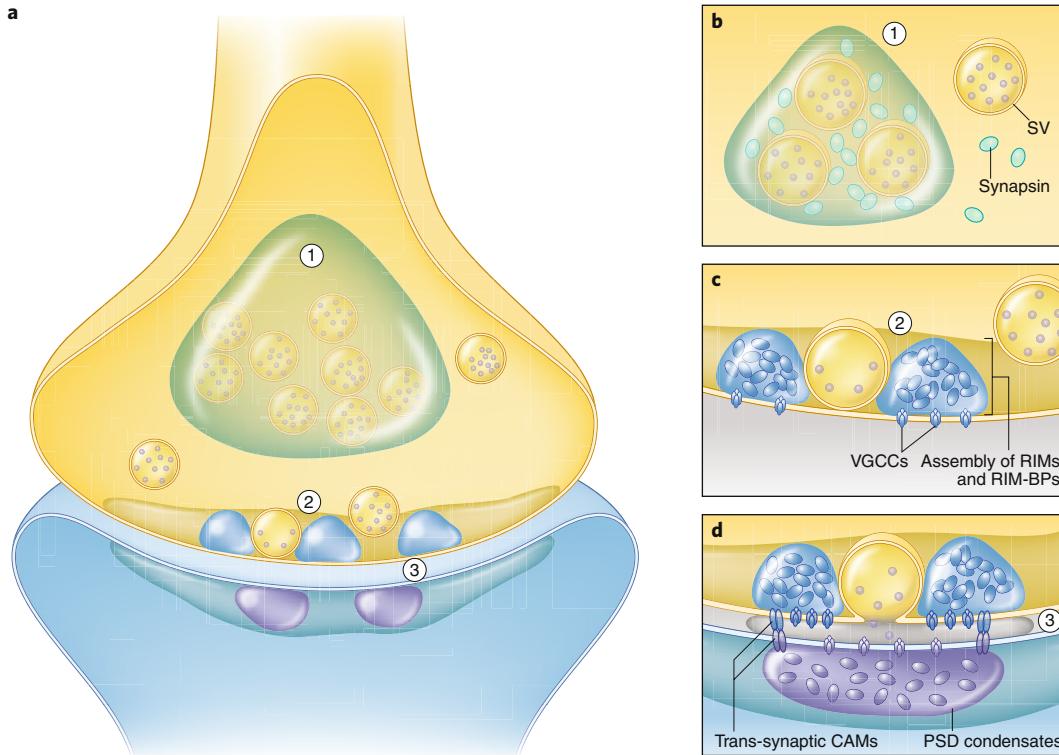


Fig. 4 | Phase separation in presynaptic boutons. **a**, Overview of the layered arrangements of the reserve pool SV phase, active zone phase and the PSD phase in a typical excitatory CNS synapse. **b**, Zoomed-in view of the reserve pool SV phase formation mediated by Synapsin phase separation and interaction between Synapsin and SVs. **c**, Enlarged view showing the active zone dense projection condensate formation and Ca^{2+} channel clustering by RIMs and RIM-BPs (and likely other presynaptic proteins such as ELKs, Munc13, Liprins, etc.). The diagram also shows possible direct interaction between the dense projection condensates with docked SVs via some still-unknown mechanism(s). **d**, An expanded view showing the trans-synaptic alignment of the presynaptic active zone condensates with the PSD condensates, likely by trans-synaptic cell adhesion molecules (CAMs).

RIM–RIM-BP mixture¹⁷. Formation of the RIM–RIM-BP–VGCC tail condensates can massively concentrate these proteins into small volumes in the condensed phase, possibly explaining how relatively low copy numbers of RIM and RIM-BP scaffolds can be highly enriched in the defined regions of active zones⁹². Furthermore, RIM and RIM-BP can efficiently cluster the VGCC tail tethered to the SLB via phase separation. The density of VGCC in the condensed phase on the SLB was estimated to be comparable to that derived from an immuno-EM-based method measuring $\text{Ca}_{2.1}$ channel clusters in synapses^{17,87}. In real synapses, other active zone proteins such as ELKs, Liprins, Piccolo and Bassoon may function together with RIMs and RIM-BPs to organize the highly dense active zone protein assembly^{10,11}.

Connecting principles of phase-separation-mediated formation of biological condensates with synaptic functions

Though phase separation is a well-known physical chemistry phenomenon and has been observed in biological systems for many years^{1,93,94}, the field of biological phase separation is at its infant stage. In theory, all polymers can undergo phase separation when proper sample conditions are met. Thus, many nucleic acids and proteins, which are polymers in nature, can undergo phase separation in solution under certain conditions. Perhaps one of the most important parameters to keep in mind when it comes to phase separation in biological systems is whether formation of condensed assemblies, by individual molecules or by molecular mixtures, occurs at physiological relevant conditions such as sample

concentrations, temperature and buffer conditions. An explosive increase in the number of studies in the last few years has uncovered many unique properties of condensed biological molecular assemblies, compared to those of molecular complexes formed in classical dilute solutions. We discuss a few of these features that are directly relevant to synaptic biology below.

Sharp concentration-dependent formation of dense molecular assemblies. A key feature of phase-separation-mediated biological condensates formation is that transition from the dilute phase to the condensed phase is hypersensitive to concentrations of molecules in the system. This means that phase separation of a system can occur once the concentration of the components in the system surpasses a certain threshold, leading to massive concentration of molecules in the condensed phase with a tiny volume. This feature offers an explanation for why subcellular signaling compartments such as PSDs or presynaptic active zones, both highly concentrated molecular assemblies existing in tiny volumes, may stably exist in neurons. The sharp concentration-dependent phase separation may also provide a potential answer to why mutation of one allele of a gene encoding a synaptic protein such as SynGAP can have such a large impact on the organization and function of the synaptic complex^{14,49}.

Another interesting feature of the condensed molecular assembly formed via phase separation is that the concentrations of each molecular component in the condensed phase remain constant once the system has reached the threshold concentration for phase separation (see Supplementary Fig. 4 of ref. ¹⁵). This observation predicts that a further

increase of the component concentration in the system would lead to a volume increase of the condensed phase. This prediction fits well with numerous experimental findings showing that overexpressing any one of the major PSD scaffold proteins (for example, PSD-95, GKAP, Shank and Homer) in neurons lead to enlargements of spine head and PSD areas. Conversely, decreasing the amount of each of these PSD scaffold proteins or specifically blocking of the PSD network formation can cause the spine head sizes in synapses to shrink^{64,95}.

Network-level properties of molecular assemblies in the condensed phase. A defining feature for forming both PSD and presynaptic active zone molecular assemblies via phase separation is that both systems require specific, multivalent interactions forming large networks of molecular organizations (Fig. 3a). This feature also predicts that the formed condense molecular assemblies may be regulated at the network level in addition to the level of individual proteins commonly understood in the dilute solutions. For example, the entire postsynaptic glutamate receptor-PSD-95-SynGAP-GKAP-Shank-Homer assembly could be regulated by targeting one single component in the network. This concept is illustrated by modulating the PSD assembly phase separation by altering the ratio of Homer1c over Homer1a in the system¹⁵. Homer1a is an alternatively spliced isoform of Homer 1c⁹⁶. Homer1a is a monomer due to its lack of the C-terminal tetramerization domain existing in Homer1c, but both Homer isoforms can bind to Shank with the same affinity¹⁵. Overexpression of Homer1a is known to cause synapse shrinkage in neurons⁹⁷. A recent study showed that sleep induces very obvious elevation of Homer1a in excitatory synapses in mice⁹⁸ and appears to correlate with the global downscaling of PSD sizes^{98,99}. Interestingly, addition of Homer1a to the NR2B tail-PSD-95-SynGAP-GKAP-Shank-Homer1c assembly mixture dispersed the condensed PSD assemblies formed either in solution or on the SLB¹⁵, illustrating that Homer1a alone can modulate the entire PSD network. In addition to increasing our understanding of the action mechanisms of the PSD network, such network-level PSD assembly modulation by a single protein may also provide new directions in searching for therapeutic strategies for psychiatric disorders caused by mutations in the genes encoding the PSD components. Instead of targeting individual proteins in the network, one might be able to modulate the entire PSD assembly by regulating the network assembly or disassembly. Due to the extreme genetic heterogeneity of psychiatric disorders, coverage of patients by a particular treatment will be narrow even if one can find an effective method targeting a specific synaptic protein. In contrast, a method effective in targeting the entire PSD network assembly will likely be able to treat a much larger population of patients.

Another implication of the network-level properties of the synaptic assemblies such as PSDs is that the regulations of a network assembly may be decoupled from direct pairwise protein–protein interactions within the network. For example, formation of the coiled coil trimer is essential for SynGAP to be enriched by PSD-95 in the condensed phase¹⁴. Therefore, mutations in the trimerization region of SynGAP were found to substantially alter PSD recruitment of SynGAP due to its impaired phase separation with PSD-95, although such mutations do not directly affect the SynGAP and PSD-95 interaction¹⁴. Additionally, phosphorylation of SynGAP in the coiled-coil region neither affects its binding to PSD-95 nor alters its trimer formation, but such phosphorylation weakens the phase separation of SynGAP with PSD-95 (our unpublished observation). Thus, some SynGAP mutations identified in patients may change the enzyme's condensation in PSD and consequently alter its synaptic function, although such mutations may not directly alter SynGAP's binding to PSD-95 or its catalytic activity^{52,54}.

Segregations and communications between different membraneless compartments. The majority of synapses in adult human brain are excitatory in nature, and excitatory synapses are mainly localized

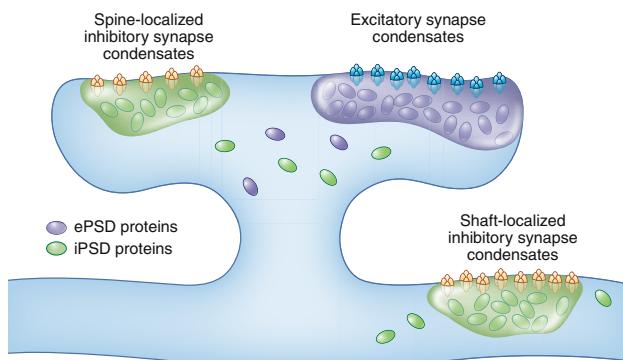


Fig. 5 | Mutual exclusion of excitatory and inhibitory PSD condensates.

A schematic model showing phase-separation-mediated segregations of excitatory and inhibitory PSDs (ePSD and iPSD, respectively) condensates within a tiny spine head. Most iPSDs are located on dendritic shafts and thus are naturally separated from ePSDs on dendritic spines.

at protrusions along dendritic spines. The majority of inhibitory synapses are formed either on cell soma or dendritic shafts. Nonetheless, a proportion of inhibitory synapses are localized on dendritic spines^{100,101}. Among those spine-localized inhibitory synapses, a considerable proportion co-exists with excitatory synapses. But inhibitory and excitatory PSDs, even within a single spine, do not inter-mix with each other¹⁰⁰, suggesting that there exists some active exclusion mechanism separating the two categories of synapses within tiny volumes of dendritic spines.

It was observed that the reconstituted excitatory PSD condensates selectively repel the inhibitory postsynaptic scaffold protein Gephyrin from entering the condensates¹⁵. Such exclusion does not occur in the dilute phase (i.e., Gephyrin mixes well with the PSD components in bulk dilute solution). It was further shown that PSD-95 plays an active role in excluding Gephyrin from the excitatory PSD condensates, as PSD-95 weakly repels Gephyrin¹⁵. This observation might be rationalized as the following: in the dilute phase, weak repulsion forces between PSD-95 and Gephyrin are not sufficient to overcome the Brownian motions of the proteins in the mixture. When the excitatory PSD components are highly concentrated in the condensed phase, the weak repulsion force is amplified and thus can cause active exclusion of Gephyrin. This is analogous, albeit in the opposite direction, to the phase separation mediated by weak protein–protein interactions of intrinsically disordered proteins^{102–108}. Such phase-separation-mediated active exclusion of one type of molecular assembly from another functionally distinct type implies how multiple condensed cellular compartments may be able to co-exist in tiny subcellular regions, such as inhibitory and excitatory PSDs in dendritic spines (Fig. 5) or the Synapsin-organized reserve pool SV phase and active zone phase formed by RIM and RIM-BP in presynaptic boutons (Fig. 4).

Future directions of phase separation in synaptic development and signaling

Forming condensed biomolecular assemblies that are not enclosed by membranes is likely a general strategy for cells to organize various cellular compartments. Historically, membrane-delimited cellular compartments have received far more attention in the field of cell biology. From an evolutionary perspective, formation of dense molecular assemblies with potential to self-replicate may have appeared earlier than membrane-enclosed systems when primitive forms of life first appeared and evolved in the vast dilute aqueous solution of oceans. If this hypothesis stands, one might expect that both the forms and functions of membraneless compartments in living cells would be much more prevalent and diverse than what

have been uncovered in the past few years. Additionally, it is almost certain that membraneless compartments and membrane-enclosed compartments can communicate with each other to regulate cellular functions. Thus, there are numerous challenges and opportunities in the field of membraneless compartments. Here we list a few that are directly relevant to synaptic biology.

1. The phase-separation-mediated synaptic assembly formations discussed here are all based on *in vitro* studies. One of the most pressing issues is to establish whether phase-separation-mediated pre- and postsynaptic condensate formation and regulation are indeed in operation in living neurons. To achieve this, we need to overcome technical barriers imposed by the unique features of synapses. Due to the awkward size of the synapse, direct observation and characterization of pre- and postsynaptic densities in the form of possible phase separation using currently available methods is not feasible. New methods will need to be developed.
2. Synapses are highly plastic and responsive to different stimulations. An important future direction is to understand how the formation of pre- and postsynaptic condensates is regulated in connection to synaptic stimulations. For example, CaMKII is the most abundant PSD component and vital for synaptic plasticity. It is extremely important to investigate how neuronal activity-induced modifications by CaMKII would modulate the PSD assembly. Given its sheer abundance in synapses, CaMKII is likely to play certain scaffold roles in addition to being an enzyme. Additionally, many other abundant proteins are known to exist in PSDs in addition to the ones already studied, and the roles of these proteins in the PSD condensates' formation and regulation are waiting to be discovered.
3. It is likely that, in the presynaptic active zones, the RIM–RIM-BP–VGCC condensates may be tethered with docked SVs, which is a form of communication between membraneless and membrane-enclosed compartments. How such communication might occur if it indeed exists? Which protein(s) may tether the two types of compartments together? Additionally, the Synapsin reserve pool SV phase should communicate with the RIM–RIM-BP–VGCC-organized readily releasable phase. How such two phases communicate with each other is not known at the time this paper was written.
4. Recent super-resolution imaging studies showed that the presynaptic RIM clusters are nicely aligned with the PSD nanoclusters, forming trans-synaptic nanocolumns for efficient synaptic transmissions⁸⁵. This observation indicates that pre- and postsynaptic density assemblies can communicate with each other even though the two types of condensates are separated by two membrane leaflets. How such distinct types of condensates communicate with each other across synaptic junctions is an interesting topic to investigate. Would trans-synaptic adhesion molecules and/or receptors also form condensed assembly in the gap region between pre- and postsynaptic membranes?
5. In addition to PSD assemblies in dendritic spines, dendritic shafts likely also contain various membraneless compartments, such as neuronal RNA granules (Fig. 2b). How such different membraneless compartments communicate and coordinate with each other in regulating synaptic development and signaling is another fertile field for future research.
6. It is tantalizing to speculate that formation of biological condensates in synapses may offer new opportunities to understand psychiatric disorders caused by mutations of genes encoding synaptic proteins and may present new avenues for developing therapeutic methods for these diseases.

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Competing interests

The authors declare no competing interests.

Additional information

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