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Phase Separation–Mediated Compartmentalization Underlies Synapse Formation and Plasticity

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Abstract

The synapse is polarized and highly compartmentalized on both its pre- and postsynaptic sides. The compartmentalization of synaptic vesicles, as well as vesicle releasing and recycling machineries, allows neurotransmitters to be released with precisely controlled timing, speed, and amplitude. The compartmentalized and clustered organization of neurotransmitter receptors and their downstream signaling enzymes allows neuronal signals to be properly received and amplified. Synaptic adhesion molecules also form clustered assemblies to align pre- and postsynaptic subcompartments for synaptic formation, stability, and transmission. Recent studies indicate that such synaptic and subsynaptic compartmentalized organizations are formed via phase separation. This review discusses how such condensed subsynaptic compartments may form and function in the context of synapse formation and plasticity. We discuss how phase separation allows for the formation of multiple distinct condensates on both sides of a synapse and how such condensates communicate with each other. We also highlight how proteins display unique properties in condensed phases compared to the same proteins in dilute solutions.

Contents

INTRODUCTION

The synapse is the structural and functional unit of neuronal connection. Morphologically, a synapse consists of pre- and postsynaptic compartments separated by a synaptic cleft. Physiologically, a synapse allows neuronal signals to transmit from one neuron to another. All information about synapse organization and function in principle is rooted in molecules. Understanding how molecules are assembled and regulated in different parts of synapses may enable us to decode how neurons are wired together to form circuits and how activities of neuronal circuits may be modulated by intrinsic and extrinsic signaling cues acting on molecules in synapses (Südhof 2017, 2018). Macrobiomolecules rarely function alone in living systems, including neuronal synapses. Instead, biomolecules interact with each other, forming functional complexes and sometimes large molecular assemblies. Different molecular complexes/assemblies are not evenly distributed but are compartmentally localized at specific subcellular regions so that different cellular processes can occur in distinct subcellular spaces (Biederer et al. 2017, Chen et al. 2020, Cole & Reese 2023, Fukata et al. 2024). The remarkable morphological and functional polarization of neurons takes the compartmentalization of molecular complexes/assemblies to the extreme. In return, the extreme molecular complex/assembly compartmentalization imposes cellular logistical challenges for how neurons manage to position and maintain specific biomolecules in distinct regions in a way that cannot be easily rationalized by the law of diffusion.

For each neuron, its synapse is a submicron-sized compartment with a volume that is thousands-fold smaller than the total cell volume (Sun & Schuman 2022). Decades of electron microscopy (EM) and more recent superresolution optical microscopy imaging studies have revealed that each synapse, though already very small, is highly polarized and further compartmentalized

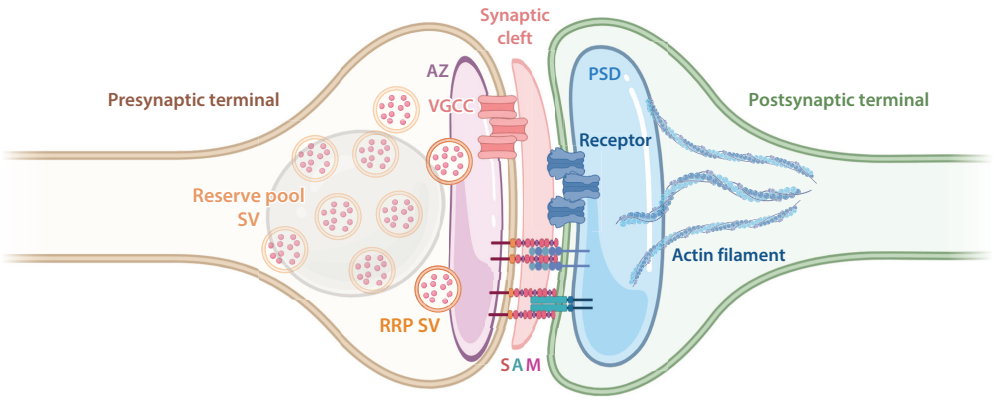

Figure 1

A synapse is highly compartmentalized. Scaffold proteins, synaptic vesicles (SVs), release machineries, receptors, and synaptic adhesion molecules (SAMs) can form distinct membraneless compartments in both the pre- and postsynaptic sides of a synapse. In the presynaptic terminal, most SVs are clustered in the reserve pool via Synapsin condensation. The active zone (AZ) condensate provides a platform for voltage-gated Ca^{2+} channel (VGCC) targeting and clustering, SV tethering, docking, priming, and fusion. On the postsynaptic side, the postsynaptic density (PSD) condensate organizes receptors and signaling enzymes into dense clusters. SAMs also form distinct compartments connecting and aligning pre- and postsynaptic condensates. Figure adapted from images created with BioRender.com.

(Dosemeci et al. 2016, Gray 1959, Hruska et al. 2018, Schikorski & Stevens 1997, Tang et al. 2016) (**Figure 1**). Under EM, electron-dense regions were observed beneath plasma membranes in both pre- and postsynaptic compartments. The electron-dense compartments on the pre- and postsynaptic sides are known as the active zone (AZ) and the postsynaptic density (PSD), respectively (Dosemeci et al. 2016, Gray 1959, Schikorski & Stevens 1997) (**Figure 1**). Interestingly, even though AZs and PSDs in synapses are not enclosed by lipid membranes, their appearance under EM is somewhat analogous to certain cellular membrane organelles. Synaptic vesicles (SVs) in presynaptic boutons are also compartmentalized, with most SVs forming a dense cluster distal to the presynaptic plasma membrane and several readily releasable pool (RRP) SVs tethered to the AZ subcompartment (Alabi & Tsien 2012, Rizzoli & Betz 2005, Siksou et al. 2007) (**Figure 1**). There is increasing evidence that synaptic adhesion molecules (SAMs), receptors, and ion channels at both pre- and postsynaptic plasma membranes are also compartmentally distributed (Choquet & Hosy 2020, Fukata et al. 2024, Nakamura et al. 2015). Such compartmentalized distributions of synaptic molecular assemblies, including clustered SVs, shape the polarized structures by placing specific biomolecules at specific and tiny subsynaptic regions amid the relatively huge volume of a neuron, allowing neuronal signals to be transmitted with precise timing, speed, and amplitude.

A striking characteristic of all these subsynaptic compartments is that molecules within and outside each of these compartments are separated by an invisible boundary and with a large concentration gradient, although these compartments do not contain enclosed physical barriers such as lipid membranes (Dosemeci et al. 2016). Such large concentration gradients of molecules within and outside the compartments cannot be explained by the classical Brownian diffusion theory for dilute solutions (Shen et al. 2023). Initially shown by using a pair of PSD proteins, postsynaptic density protein 95 (PSD-95) and synaptic Ras GTPase-activating protein (SynGAP) (Zeng et al. 2016), and later demonstrated by numerous other molecular systems in both pre- and postsynaptic subcompartments (for examples, see Milovanovic et al. 2018, Wu et al. 2019, Zeng et al. 2018), an autonomous molecular demixing process at mesoscopic scale known as liquid-liquid phase separation (referred to as phase separation from here on) was demonstrated to be a molecular mechanism underlying the formation of these subsynaptic compartments. In

fact, phase separation is being recognized as a fundamental principle for concentrating biological macromolecules into distinct compartments in subcellular spaces in cells (Banani et al. 2017, Lyon et al. 2021, Zhang et al. 2020). Since the cellular compartments formed by phase separation typically contain highly concentrated biomolecules and each formed compartment has specific biological functions analogous to membrane-based organelles, the condensed molecular assemblies formed via phase separation are often referred to as biological condensates or membraneless organelles. How does phase separation–mediated compartmentalization contribute to synapse formation, organization, and plasticity? How can multiple subcompartments coexist in the same tiny space, such as in a presynaptic bouton or a postsynaptic spine protrusion, even though these compartments are not demarcated by lipid membranes? How are the subcompartments subject to dynamic regulations in response to synaptic activities? How may phase separation contribute to dynamic processes in presynaptic boutons such as SV movements, fusion, and recycling? How do pre- and postsynaptic subcompartments communicate and coordinate with each other for synaptic formation and plasticity? We only briefly summarize some of the findings from recent studies on this topic, and readers are referred to several previous reviews for more details (Chen et al. 2020, Feng et al. 2021, Hayashi et al. 2021, Wu et al. 2020).

SCAFFOLD PROTEINS ORGANIZE PRE- AND POSTSYNAPTIC SUBCOMPARTMENTS

During embryonic and postnatal development, neurons differentiate, migrate, and mature to grow axons and dendrites. Upon initial contact between axons and dendrites, mediated by SAMs, a nascent synapse starts to form (Südhof 2018, 2021). Axons and dendrites form prototypes of presynaptic organization and postsynaptic specialization, respectively. Functional synapse formation requires an activity-dependent maturation step, during which pre- and postsynaptic specializations develop into functional pre- and postsynaptic compartments, a process involving the addition of SVs and vesicle clustering, releasing and recycling proteins in presynaptic boutons, neurotransmitter receptors and their downstream signaling, and scaffolding proteins in postsynaptic compartments, as well as actin cytoskeletons in both compartments.

Intracellular scaffold proteins in both pre- and postsynaptic compartments have been demonstrated to form condensates beneath synaptic plasma membranes via phase separation. PSDs are condensed protein/enzyme mixtures responsible for clustering neurotransmitter receptors and for transducing signals received by the receptors. Biochemically purified PSDs are highly stable (e.g., resistant to detergent solubilization), yet PSDs can be rapidly and dynamically regulated by synaptic activities. PSD proteins, including PSD-95, SynGAP, SAP90/PSD-95-associated protein (SAPAP), SH3 and multiple ankyrin repeat domains protein 3 (Shank3), and Homer1, interact with each other, forming large molecular networks with an emergent property of autonomously demixing into two distinct phases in solution and on the surface of lipid membranes (Wu et al. 2024, Zeng et al. 2018). The condensed phase of the PSD mixtures can cluster glutamate receptors and enrich enzymes. The presynaptic AZ is functionally defined as the hotspot for neurotransmitter release and structurally characterized by the condensed proteinaceous materials attached to the presynaptic plasma membrane. Similarly, multiple AZ scaffold proteins, including Rab3-interacting molecule (RIM), RIM binding protein (RIMBP), ELKS [named for the rich content of glutamic acid (E), leucine (L), lysine (K), and serine (S)], Liprin-α, and Munc13, have been shown to phase separate in solution and on membrane surfaces to form condensed molecular networks capable of clustering voltage-gated Ca^{2+} channels (VGCCs). We have systematically summarized these findings in earlier reviews (Chen et al. 2020; Wu et al. 2020, 2023) and do not provide further details here.

Organization of Multiple Subcompartments in Presynaptic Boutons

Despite its exceedingly small volume, each presynaptic bouton is further compartmentalized. Most SVs are clustered as a reserve pool by proteins such as Synapsin and its binding partners (Intersectin, GRB2, Synaptophysin, etc.) via coacervation of SVs with the Synapsin protein condensates (Milovanovic et al. 2018, Park et al. 2021, Sansevrino et al. 2023, Song & Augustine 2023). RRP SVs instead coat the surface of the AZ condensates (Wu et al. 2021). Thus, the distinct protein condensates formed by different sets of proteins allow partitioning of chemically similar SVs into functionally distinct pools (**Figure 2*a***).

Even though molecular components within the AZ can interact with each other, AZs are further compartmentalized. For example, Munc13 and VGCC have been suggested to exist in distinct subcompartments within AZs, and their distances correlate with synaptic transmission efficiency (Rebola et al. 2019). In strong synapses, such as those formed between stellate cells, the Munc13-containing subcompartments are adjacent to the VGCC clusters, ensuring strong neurotransmission. In contrast, in weak synapses, such as those formed between granule cells and Purkinje cells, Munc13 subcompartments are relatively far away from the VGCC clusters, resulting in weaker coupling of action potential–induced Ca^{2+} entry with the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) release machinery and consequent weaker neurotransmission (Rebola et al. 2019). In addition, Munc13 and VGCC are transported to the AZ via different mechanisms (Rizalar et al. 2023). The segregation of Munc13 and VGCCs is counterintuitive when viewed from the perspective of the canonical molecular interaction framework as both proteins directly interact with RIM through different regions (Emperador-Melero & Kaeser 2020). Additionally, when Munc13 and VGCCs were forced to interact with each other by fusing the Munc13 binding (zinc finger) domain of RIM to VGCC, the requirement of RIM for SV release could be bypassed (Tan et al. 2022), indicating a role of RIM in functionally connecting these two proteins together. A possible solution to reconcile these seemingly contradicting observations (i.e., physical separation of Munc13 and VGCCs in AZs and direct connection of the two proteins by RIM) is that RIM can organize distinct subcompartments. A recent study provided direct evidence supporting such a hypothesis by showing that in HEK293T cells and in mouse hippocampal neurons, VGCC and Munc13 are individually organized by RIM into two distinct molecular assemblies: a VGCC nanocluster likely organized by the RIM/RIMBP condensate and a SV priming/release nanodomain containing RIM, Munc13, Liprin-α, and receptor protein tyrosine phosphatases (RPTPs) (Emperador-Melero et al. 2024) (**Figure 2*a***). Consistently, genetic ablation of all four *liprin-α* isoforms led to dramatic impairment of priming machinery (RIM and Munc13) accumulation, but the localization of VGCC was barely or only mildly affected (Emperador-Melero et al. 2024, Marcó de la Cruz et al. 2024). Liprin-α proteins have been shown to undergo phase separation, which is crucial for the recruitment and assembly of many other downstream scaffold proteins and maturation of the presynaptic bouton (Emperador-Melero et al. 2021; Liang et al. 2021; McDonald et al. 2020, 2023).

PSD Subcompartmentalization and Dynamic Regulation: Synaptic Activity–Dependent Fusion and Fission of PSD Subcompartments

The structural dynamics of PSD condensates functionally correlate with synaptic strength and plasticity. The PSD area is nearly linearly correlated with the activity of a synapse (Borczyk et al. 2019, Holler et al. 2021). Earlier EM studies have already characterized the heterogeneity of the PSD compartments. There is a denser compartment right beneath the postsynaptic plasma membrane called the PSD core, and a looser compartment located deeper into the spine known as the PSD pallium (Dosemeci et al. 2016). Upon activation, the PSD of a synapse

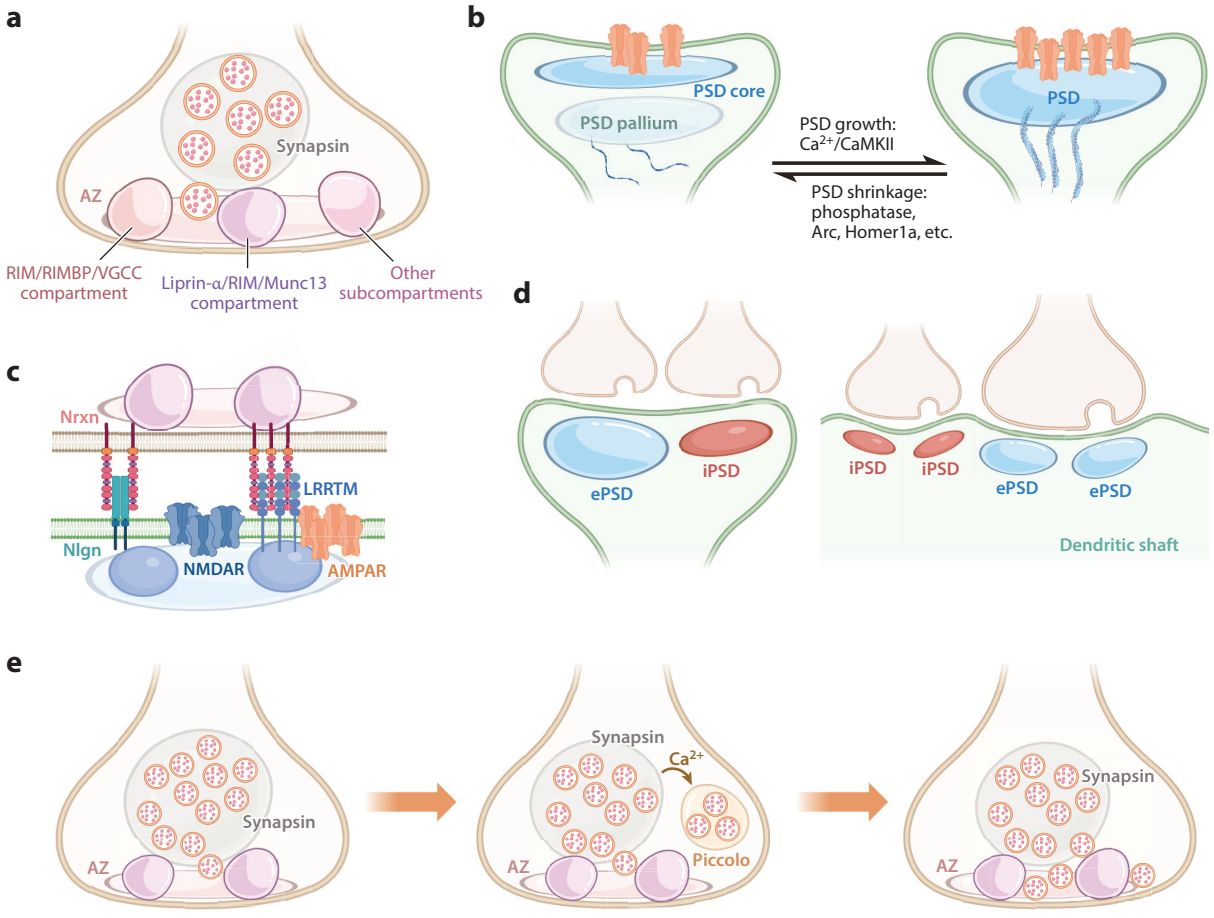

Figure 2

Modular features and regulation of subsynaptic compartments. (*a*) Multiple subcompartments in presynaptic boutons. Synapsin and active zone (AZ) proteins phase separate to organize synaptic vesicles (SVs) into a reserve pool and a readily releasable pool. Within the AZ, multiple subcompartments organized by different sets of proteins coexist as distinct nanoclusters. Rab3-interacting molecule (RIM) can simultaneously organize the Ca^{2+}-generating nanodomain composed of RIM/RIM binding protein (RIMBP)/voltage-gated Ca^{2+} channel (VGCC), the SV priming machinery formed by RIM/Liprin-α/Munc13, and possibly other subcompartments. (*b*) Multiple subcompartments and dynamic regulation in the postsynaptic density (PSD). The PSD is assembled with a membrane proximal core condensate and a membrane distal pallium condensate. Activation of calcium/calmodulin-dependent protein kinase II (CaMKII) by Ca^{2+}-CaM triggers growth of the PSD by promoting fusion of the PSD core condensate with the PSD pallium condensate. Phosphatase can reverse this process. PSD condensates can bundle F-actin and promote spine growth. The increase of Arc and Homer1a can disperse the PSD condensate and consequently weaken synapses. (*c*) Synaptic adhesion molecules (SAMs) [e.g., Neurexin (Nrxn), Neuroligin (Nlgn), Leucine-rich repeat transmembrane neuronal protein (LRRTM)] mediate modular alignment between pre- and postsynaptic compartments, forming nanocolumns. (*d*) Phase separation allows multiple subcompartments to coexist. In dually innervated spines (*left*), excitatory PSD (ePSD) and inhibitory PSD (iPSD) each organize specific subcompartments within the tiny spine heads. In dendrites of interneurons, ePSD/ePSD, iPSD/iPSD, and ePSD/iPSD are all well separated. (*e*) SV transportation between subcompartments within a presynaptic bouton. Reserve pool SVs are maintained by the Synapsin compartment. Upon Ca^{2+} stimulation, Piccolo extracts SVs from the Synapsin compartment and delivers vesicles to the AZ compartment to refill the readily releasable pool emptied by action potentials. Figure adapted from images created with BioRender.com.

becomes larger, thicker, and denser, though the underlying molecular mechanisms are largely unknown. A recent study revealed that the PSD core condensate formed by PSD-95, SynGAP, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and the PSD pallium condensate formed by Shank3, Homer1, and SAPAP are segregated, forming two distinct subcompartments. Activation of calcium/calmodulin-dependent protein kinase II (CaMKII) causes fusion of the two PSD subcompartments into one larger and denser condensate, reminiscent of the synaptic activity–induced PSD structural long-term potentiation (LTP) (Wu et al. 2024) (**Figure 2*b***). Mechanistically, SAPAP phosphorylation dramatically increases its affinity toward PSD-95 (Zhu et al. 2017), causing the PSD core condensate network and PSD pallium condensate network to fuse into a single larger network (Shen et al. 2023). Dephosphorylation of SAPAP triggers fission of the PSD condensate into two subcompartments: the PSD core condensate and the PSD pallium condensate. The reversible phosphorylation-regulated PSD condensate growth and shrinkage suggest that, analogous to membrane-based organelles, membraneless organelles formed via phase separation can also undergo regulated fusion and fission. In addition, PSD condensates are able to promote actin bundling without requiring canonical actin regulatory proteins (Chen et al. 2023) (**Figure 2*b***). Both Homer-mediated actin binding and PSD condensation are essential for actin bundling. Specific amino acid changes in Homer weakening its actin binding impaired actin bundling. The immediate early gene product Homer1a, which binds to actin like the longer Homer isoforms but can actively downregulate PSD condensate formation (Zeng et al. 2018), also impaired PSD condensate-promoted actin bundling and spine growth. Another immediate early gene product, Arc, specifically binds to transmembrane AMPAR regulatory proteins (TARPs), which antagonizes the interaction between PSD-95 and AMPAR and disperses AMPARs from the PSD condensate both in vitro and in living neurons (Chen et al. 2022), explaining why Arc can effectively modulate synaptic strength in a protein concentration–dependent manner.

Apart from the scaffold proteins, AMPARs and *N*-methyl-D-aspartate receptors (NMDARs) are also organized into condensed nanoclusters (Choquet & Hosy 2020, Ferreira et al. 2020, Fukata et al. 2024). Phase separation of PSD scaffold proteins contributes to each receptor's compartmentalization (Cai et al. 2021, Zeng et al. 2019). However, AMPAR and NMDAR clusters are not colocalized. Superresolution imaging studies uncovered that one relatively stable NMDAR nanocluster is surrounded by several dynamic AMPAR nanoclusters (Goncalves et al. 2020, Jeyifous et al. 2016). Mechanistically, activation of CaMKII can cause glutamate receptor subcompartmentalization. In this case, activated CaMKII acts as a scaffold protein to bind to NMDARs and forms a CaMKII/NMDAR condensate that is separated from the AMPAR-containing PSD condensate (Hosokawa et al. 2021).

Synaptic Activity–Regulated AZ and PSD Compartment Alignment: From Nanoclusters to Nanocolumns

In each synapse, the AZ and PSD are within a lateral length of several hundred nanometers and are near perfectly aligned (**Figure 2*c***). The AZ and PSD components can individually organize into unique subcompartments, and these subcompartments are not randomly distributed but instead transsynaptically linked (Biederer et al. 2017, Cole & Reese 2023, Tang et al. 2016). The structural alignment between these subcompartments is functionally correlated with synaptic transmissions. This is because AMPARs bind to glutamate with low affinity, so the receptor density and distance to the transmitter release site are critical for AMPAR opening probability (Choquet & Hosy 2020, Greger et al. 2017, Liu et al. 1999). Superresolution fluorescence microscopic studies revealed that the AZ (viewed by RIM) and PSD (viewed using PSD-95 or AMPAR) form nanoclusters

approximately 80 nm in diameter (Fukata et al. 2013, MacGillavry et al. 2013, Nair et al. 2013, Tang et al. 2016). Interestingly, these nanoclusters are preferentially aligned, forming the so-called transsynaptic nanocolumns (Tang et al. 2016). The formation of AZ and PSD nanodomains and their alignment into nanocolumns have been directly visualized by EM tomography studies using purified synaptosomes (Martinez-Sanchez et al. 2021) or by directly imaging synapses in cultured neurons (Cole & Reese 2023, Held et al. 2024). Apart from the RIM nanoclusters, Munc13 also forms nanoclusters in the AZ (Dharmasri et al. 2024, Rebola et al. 2019, Sakamoto et al. 2018). Interestingly, the VGCC-containing RIM nanoclusters do not directly overlap with the Munc13-containing nanoclusters (Emperador-Melero et al. 2024)—a finding consistent with an EM tomography study showing that SVs in the AZ region are not preferentially aligned with the AZ-PSD nanocolumns (Held et al. 2024). In addition to facilitating synaptic transmission, the alignment between the AZ and PSD may also contribute to synaptic stability. Simultaneous knockout of RIM and RIMBP leads to loss of the AZ, accompanied by the expansion of PSD (Acuna et al. 2016). The transsynaptic nanocolumn organization is regulated. Upon LTP induction, AZ nanodomains become better aligned with PSD nanodomains (Clavet-Fournier et al. 2024, Tang et al. 2016).

Phase Separation Allows Multiple Subcompartments to Coexist in Tiny Pre- and Postsynaptic Spaces

Neither the presynaptic bouton nor the postsynaptic spine protrusion of a synapse is enclosed by physical barriers. How can multiple subcompartments with distinct molecular components coexist in each of these tiny spaces? Put another way, why do these molecules not mix with each other following the law of diffusion? The concept of biomolecular phase separation provides a mechanistic explanation of the coexistence of multiple condensed molecular assemblies in an open space in 3D cytoplasm or on the surface of 2D membranes. Phase separation–mediated biological condensate formation is governed by multivalent molecular interactions in each condensate, and such multivalent interaction causes molecules in the condensate to form a percolated network (Mittag & Pappu 2022). The percolated network in each condensate, which exists in a crowded cellular milieu, including synapses, is formed by a combination of strong and specific interactions as well as weak interactions via intrinsically disordered sequences between biomolecules (Rana et al. 2024, Shen et al. 2023, S. Zhu et al. 2024). Breaking distinct networks in each condensate is an energy-demanding process. Thus, for a two-condensate system, if the entropic gain from molecular mixing cannot compensate for the energy costs of breaking the molecular networks, the two condensates will be demixed. This concept was recently directly tested in addressing why inhibitory PSDs (iPSDs) and excitatory PSDs (ePSDs) in neurons are always segregated (S. Zhu et al. 2024) (**Figure 2*d***). Both the ePSD and iPSD are condensed molecular assemblies formed via phase separation (Bai et al. 2021, Zeng et al. 2018). The compartmentalization of the ePSD and iPSD is taken to an extreme in a category of excitatory postsynaptic spine protrusions in which a single spine is simultaneously innervated by an excitatory input and an inhibitory input (**Figure 2*d***). By mixing five ePSD proteins and four iPSD proteins in vitro, S. Zhu et al. (2024) found that the formed ePSD and iPSD condensates are naturally demixed and such demixing requires specific multivalent interactions within the ePSD protein complex network and the iPSD protein network, respectively. The segregation of ePSD and iPSD condensates could be recapitulated in heterologous cells. Strikingly, the separation of the ePSD network and the iPSD network is so robust that even installing a forced interaction between iPSD scaffold Gephyrin and ePSD scaffold PSD-95 with a nanomolar binding affinity nanobody could not mix the two condensates in vitro and in living neurons. This study indicates that, instead of diffusion-governed spontaneous mixing in dilute solutions, demixing is a naturally occurring process for biomolecules in condensates formed via phase separation. Both the complexity (e.g., levels of valency) and stability (e.g.,

the binding affinities between each pair of the interacting molecules) of the percolated network can tune the pattern and robustness of multiphase organizations in cells. This study also provides an explanation as to why numerous biological condensates can coexist as distinct organelle-like structures in different subcellular regions of cells, including synapses of neurons (Boeynaems et al. 2018, Chen et al. 2020, Feng et al. 2021, Hayashi et al. 2021, Shin & Brangwynne 2017, Wu et al. 2020, Zhao & Zhang 2020).

Synaptic Protein Abundance Paradox: Enzymes Versus Scaffolds

A peculiar feature of PSD molecular components is that some enzymes, such as CaMKII and SynGAP, are enriched within the PSD with extremely high abundance (higher than or at comparable levels to the major scaffold proteins in the PSD). For example, the abundance of CaMKII in the PSD is over tenfold higher than that of PSD-95 (Bayer & Schulman 2019, Sheng & Kim 2011). It has been suggested for a long time that, in addition to functioning as a kinase, CaMKII also plays certain structural roles in synapses (Liu et al. 2021, Yasuda et al. 2022). A series of recent studies provided direct evidence supporting the structural roles of CaMKII in synapses (Cai et al. 2021, 2023; Chen et al. 2024; Hosokawa et al. 2021; Tullis et al. 2023). Multivalent interaction-mediated molecular network formation is the most basic molecular underpinning in driving phase separation of biomolecular systems. CaMKII is a dodecamer/tetradecamer, and thus the enzyme is an ideal scaffold for facilitating synaptic condensate formation. Indeed, it was shown that CaMKIIα forms a condensate upon binding to different partners in a Ca^{2+}-regulated manner (Cai et al. 2021, Hosokawa et al. 2021). In the absence of Ca^{2+}, the conformationally closed and autoinhibited apo-CaMKIIα binds to Shank3, and the complex can phase separate to form condensates at protein concentrations well below those found in synapses (Cai et al. 2021). Binding of Ca^{2+}-CaM to CaMKII activates and concomitantly opens the conformation of the enzyme. The conformationally open CaMKII binds to and undergoes phase separation together with NMDAR subtype 2B (GluN2B) (Cai et al. 2021, Hosokawa et al. 2021). These findings suggest a model where, under the basal condition, CaMKII can be enriched in the lower layer of the PSD condensate containing SAPAP, Shank3, and Homer via binding to Shank3. Upon synaptic activation, Ca^{2+} activates CaMKII and promotes its translocation to the PSD core condensate containing GluN2B and PSD-95. Such a model fits well with EM and fluorescence imaging–based observations showing that, upon synapse activation, CaMKII undergoes an obvious shift from spine cytoplasm or the PSD pallium to the PSD core (Shen & Meyer 1999, Tao-Cheng 2020).

The structural role of CaMKII in supporting synaptic LTP, via binding to GluN2B, has been elegantly demonstrated by two very recent complementary studies (Chen et al. 2024, Tullis et al. 2023). Tullis et al. (2023) developed novel ATP-competitive CaMKII inhibitors that can interfere with kinase activity but with minimal impact on GluN2B binding. They demonstrated that as long as the GluN2B binding role of CaMKII is retained, the kinase activity of the enzyme is not required for LTP induction (Tullis et al. 2023). Using various forms of CaMKII mutants and electrophysiology studies, Chen et al. (2024) showed that a CaMKII mutant with its kinase activity removed (i.e., kinase dead) but with its autoinhibitory segment in the open conformation can fully support both basal synaptic AMPAR activity and LTP. Chen et al. further showed that, if GluN2B was mutated to lose its binding to CaMKII, even fully activated CaMKII could not support basal synaptic AMPAR activity or induce LTP (Chen et al. 2024). Two major isoforms of CaMKII, CaMKIIα and CaMKIIβ, exist in synapses, and both isoforms can bind to GluN2B with comparable affinities (Bayer et al. 2006, Cai et al. 2023). However, the two isoforms of CaMKII play distinct roles in synaptic activities. In hippocampal neurons, CaMKIIα, but not CaMKIIβ, rescues the deficits that result from deleting both isoforms of CaMKII (Incontro et al. 2018). A recent study revealed that CaMKIIα, which has a shorter linker connecting the kinase domain and

the hub domain compared to that of CaMKIIβ, has a higher capacity to form condensed clusters with GluN2B via phase separation (Cai et al. 2023), again highlighting the scaffolding role of CaMKII in synaptic functions.

SynGAP, a Ras GTPase-activating protein (GAP), is another example. The concentration of SynGAP in the PSD is comparable to that of PSD-95 (Sheng & Kim 2011). Loss of one copy of SynGAP causes neurodevelopmental disorders, including intellectual disability, autism, epilepsy, and others (Gamache et al. 2020), indicating the importance of SynGAP concentration for its synaptic functions. In the initial discovery of the PSD phase separation study, it was found that both the formation of the trimer and the concentration of SynGAP are critical for the phase separation of the PSD-95/SynGAP complex (Zeng et al. 2016), implying that SynGAP also plays a structural role in the PSD. A recent study directly examined the structural role of SynGAP in LTP using *Syngap1* knockin mice carrying a catalytically inactive mutant (Araki et al. 2024). Surprisingly, it was found that the GAP activity of SynGAP is not required for mice viability, LTP, and working memory. Mechanistically, SynGAP competes with AMPAR for binding to and forming condensates with PSD-95, and thus the dosage of SynGAP instead of its GAP activity can directly regulate synaptic clustering of AMPAR (Araki et al. 2024).

PHASE SEPARATION IN SV TRANSPORT WITHIN PRESYNAPTIC BOUTONS AND IN SV RELEASE

The compartmentalization of SVs into functionally distinct subpopulations in presynaptic boutons immediately raises several questions, such as, How are SVs moved directionally from one subcompartment to another? How are such directional movements regulated? Why are such an abundant number of SNARE-fusion machinery–related proteins required for SVs to fuse with or be recycled from presynaptic plasma membranes? Studies from the past few years have indicated that the phase separation of proteins together with SVs is involved in these processes. Several recent reviews have covered how phase separation mediates SVs' compartmentalization and allocation (McDonald & Shen 2021, Reshetniak & Rizzoli 2021, Sansevrino et al. 2023, Wu et al. 2023); here we only discuss some of the most recent progress on these topics.

SV Transportation Between Subcompartments Within the Bouton: Phase Separation–Mediated Short-Distance Vesicle Transport

Docking (or coating) of SVs to the surface of the AZ is a prerequisite for subsequent fusion of SVs with plasma membranes. The number of docked SVs in each presynaptic bouton is proportional to the size (or the surface area) of the AZ, so that only a few SVs are docked to the AZ (Schikorski & Stevens 1997, Siksou et al. 2007). As such, these docked SVs are collectively regarded as the RRP SVs, including those primed for fusion (Imig et al. 2014, Kaeser & Regehr 2017). Vesicles must be transported from the reserve pool to the AZ surface to refill the RRP after action potential–induced transmitter release (Alabi & Tsien 2012, Chanaday et al. 2019, Reshetniak & Rizzoli 2021, Südhof 2004). Such directional SV movements involve transport of SVs between two condensates organized by distinct sets of proteins (Milovanovic et al. 2018, Park et al. 2021, Wu et al. 2021) and separated by only a few hundred nanometers at most. Such short-distance vesicle transport is prevalent in other subcellular regions (Gomez-Navarro & Miller 2016, Lee et al. 2004) but with enigmatic mechanisms. A recent study uncovered that, in response to Ca^{2+} and mediated by the giant scaffold protein Piccolo, SVs can be specifically transported from the Synapsin-clustered condensate to the surface of the AZ protein condensate (Qiu et al. 2024). In the absence of Ca^{2+}, Piccolo and Synapsin coacervate with SVs, forming a clustered pool. Binding of Ca^{2+} to the C-terminal C2A domain of Piccolo increases the affinity between SV and Piccolo

(Garcia et al. 2004, Gerber et al. 2001), causing Piccolo and SVs to form a distinct condensate separated from the Synapsin condensate. Thus, in response to Ca^{2+}, Piccolo can extract SVs from the reserve pool maintained by Synapsin. Piccolo specifically binds to the AZ proteins RIMBP and ELKS with high affinities so that Piccolo is preferentially partitioned to the AZ condensate, but the Piccolo-extracted SVs are deposited on the surface of the AZ condensate. Thus, the pool of SVs clustered by Ca^{2+}-bound Piccolo can be viewed as a transient pool reminiscent of the rather imaginary shuttling pool SVs described in the literature (Kusick et al. 2022, Mochida 2020, Tran et al. 2022). An emerging picture is that SVs can phase separate with multiple presynaptic scaffold proteins, and the partitioning of SVs in these distinct condensates is regulated by Ca^{2+}, thus allowing directed SV movements from the reserve pool to the docked pool for final fusion when a synapse is repeatedly stimulated (Qiu et al. 2024) (**Figure 2*e***). It is worth noting that such Ca^{2+}-regulated vesicle transport does not need molecular motors or cytoskeletons, in accordance with EM observations showing that the bouton space connecting the reserve pool SVs and the AZ is devoid of cytoskeletons (Fernández-Busnadiego et al. 2010, Held et al. 2024). The study by Qiu et al. (2024) provides a proof-of-concept principle that short-distance vesicle transport, a process likely occurring in many other subcellular regions such as between Golgi stacks and from the endoplasmic reticulum (ER) to an ER-Golgi intermediate compartment, can be achieved via phase separation of vesicles with proteins. It appears that phase separation is an ideal process for mediating short-distance vesicle transport in cells. It should be noted that for movements of vesicles over a long distance, such as axonal transport of SVs, molecular motor-mediated transport is the dominant process (Cason & Holzbaur 2022).

Phase Separation in SV Fusion and Endocytosis: Enhancing Vesicle Fusion/Fission Speed and/or Specificity by Phase Separation

SNARE proteins and their associated proteins are direct executors of membrane fusions not only for SVs but also for other cellular vesicles (Jahn & Scheller 2006, Jahn et al. 2024). SNARE proteins are one of the most abundant proteins in the presynaptic bouton, with each SV containing as many as 70 copies of SNARE proteins (Takamori et al. 2006, Wilhelm et al. 2014). Only a few pairs of SNARE molecules at most are sufficient to mediate the final step of membrane fusion (Held et al. 2024, Heo et al. 2021, Montecucco et al. 2005). Analogous to CaMKII and SynGAP discussed above, the high copy number of SNARE proteins per vesicle implies that they have structural roles in addition to forming stable trans-SNARE complexes required for zipping SV and plasma membranes together. An early in vitro reconstitution assay demonstrated that the SNARE proteins syntaxin1, SNAP25, and synaptobrevin2 are the minimal components for membrane fusion (Weber et al. 1998), though such basic fusion machinery is much too slow to support efficient neurotransmissions. Addition of Munc18, Munc13, Synaptotagmin1, and Complexin1 can dramatically speed up the fusion reaction (Lai et al. 2017, Ma et al. 2013, Rizo 2022, Zhang & Hughson 2021); again, the concentrations of proteins added are much higher than the vesicle concentration in these fusion assays. Phase separation–mediated protein/enzyme condensation is known to regulate the speed and fidelity of chemical reactions (Du & Chen 2018, Peeples & Rosen 2021). Recent studies have started to reveal that condensation of these SV fusion–related proteins may serve as an alternative mechanism to accelerate the speed at which SVs fuse with plasma membranes. It has been shown that SNARE proteins can form high-order assemblages (Han et al. 2017, Mertins et al. 2021, Rothman et al. 2017). Synaptotagmin1 was recently shown to undergo Ca^{2+}-regulated phase separation, and the formed condensate can enrich SNARE proteins and consequently may impact SNARE assembly and SV fusion (Courtney et al. 2021, Mehta et al. 2024, M. Zhu et al. 2024). Synaptophysin, the second most abundant protein on SVs and a binding partner of the most abundant v-SNARE Synaptobrevin2/VAMP2 (Adams et al.

2015, Becher et al. 1999, C. Wang et al. 2024a), has been shown to modulate phase separation of Synapsin and SV compartmentalization as well as to chaperone SNARE complex assembly (Park et al. 2021, 2024). Synaptobrevin2/VAMP2, via its juxtamembrane region, directly interacts with α-synuclein to regulate α-synuclein phase separation, SV clustering, and SNARE complex assembly (Agarwal et al. 2024, C. Wang et al. 2024b). Going forward, it will be important to obtain more direct evidence showing how phase separation can specifically regulate both the speed and timing of SNARE-mediated SV fusion processes.

Phase separation has also been implicated in regulating the speed of SV endocytosis (Imoto et al. 2023, 2024). Compensatory ultrafast endocytosis following SV exocytosis is very rapid and thus requires the rapid actions of Dynamin GTPase to pinch off endocytic pits (Azarnia Tehran & Maritzen 2022; Watanabe & Boucrot 2017; Watanabe et al. 2013a,b). Imoto et al. (2023) showed that a specific isoform of Dynamin (Dynamin 1xA) is required for ultrafast endocytosis by forming condensates at the endocytic zone together with BAR domain–containing membrane binding protein Syndapin1 via direct binding. Disruption of the interaction between Dynamin1 and Syndapin1 or disruption of the BAR domain–mediated membrane binding of Syndapin1 led to stalled endocytosis of recycling SVs. Formation of the Dynamin1 and Syndapin1 condensate allows a high concentration of Dynamin1 to be deposited on the membrane surface at the endocytic site so that the slow recruitment of Dynamin1 by passive diffusion can be bypassed and preassembled endocytic machinery can be stably built on-site for ultrafast endocytosis. In addition to Syndapin1, Dynamin1 also binds to multiple SH3 domain–containing proteins via its C-terminal disordered proline-rich motifs, such as Endophilin A1 and Intersectin1, which are also suggested to be involved in phase separation–regulated endocytosis (Gowrisankaran et al. 2020, Mondal et al. 2022, Yoshida et al. 2023). Interestingly, many of these proteins also contain a BAR domain, which is known to shape and modulate membrane curvatures (Mondal & Baumgart 2023, Shishkov et al. 2023, Yuan et al. 2021). Whether and how these proteins regulate Dynamin-mediated endocytosis require further investigation.

SYNAPTIC ADHESION MOLECULE COMPARTMENTALIZATION IN SYNAPSE FORMATION AND PLASTICITY

SAMs are critical for nascent synapse formation, synapse maturation, and synaptic plasticity. This topic has been extensively reviewed, and we refer readers to some of these recent reviews (Sanes & Zipursky 2020; Südhof 2018, 2021; Verpoort & de Wit 2024). Here we cover some of the recent studies showing how SAMs function in organizing compartmentalized pre- and postsynaptic protein assemblies during synapse formation and function.

A general feature for most SAMs (except for the nontransmembrane SAMs that function through binding to transmembrane SAMs) is that each contains a large and folded extracellular architecture for binding to cognate SAMs and a short and unstructured cytoplasmic tail. The extracellular part of SAMs can be extremely diverse due to both the existence of multiple isoforms within the same family and the extensive alternative splicing of each isoform. The short intracellular tails of SAMs are much less diverse. Because a synapse is intrinsically asymmetric, SAMs in presynaptic membranes are largely different from those in postsynaptic membranes, and most of the transsynaptic interactions between SAMs are heterophilic. Also, because presynaptic boutons are less heterogenous than postsynaptic specializations in the nervous system, there are many more categories of postsynaptic SAMs than presynaptic SAMs. In theory, numerous unique combinations of transsynaptic SAM complexes can form to organize vast numbers of specific types of synapses (and hence circuits). In contrast, SAM-mediated intracellular signaling pathways in both pre- and postsynaptic compartments are much less diverse, mirroring the limited diversity of presynaptic vesicle–releasing/recycling machineries or the postsynaptic signaling complexes

organized in the form of PSDs. Thus, the vast numbers of distinct synapses formed via very diverse transsynaptic SAM complexes converge onto limited numbers of signaling machineries in pre- and postsynaptic termini.

Both the PSD condensate and the AZ assembly discussed above are possibly anchored to synaptic plasma membranes by SAMs or directly by tails of neurotransmitter receptors. Such anchoring is mediated by binding of the intracellular domain of SAMs to scaffold proteins in the pre- or postsynaptic protein condensates (Biederer et al. 2017, Jang et al. 2017, Kurshan & Shen 2019). For example, leukocyte common antigen-related receptor protein tyrosine phosphatase (LAR-RPTP) or Neurexin SAMs on presynaptic membranes bind to the Liprin-α family or other scaffold proteins to organize AZ formation as well as SV clustering (Emperador-Melero et al. 2024, Kurshan et al. 2018, Marcó de la Cruz et al. 2024, Schaan Profes et al. 2024, Takahashi & Craig 2013, Um & Ko 2013). In the PSD, TARP family proteins can directly bind to PSD-95, and Latrophilin3 can bind to Shank3 (S. Wang et al. 2024, Zeng et al. 2019). Both interactions allow anchoring of PSD condensates right beneath the postsynaptic membranes. Besides synaptic membrane anchoring, binding of transmembrane proteins such as SAMs to synaptic condensates can have multiple implications. First, recruiting cytoplasmic proteins onto the membrane surface converts the protein-protein interactions in 3D space in solution to a 2D membrane plane, which can dramatically reduce the threshold concentration for phase separation to occur (Wu et al. 2019; Zeng et al. 2018, 2019). Many SAMs are multimers, and the formation of a transsynaptic SAM complex further promotes clustering of SAMs (Shipman & Nicoll 2012, Tanaka et al. 2012). Furthermore, binding to the synaptic condensates can in return enhance the clustering of SAMs (Xie et al. 2020). Since phase separation–mediated condensate formation is extremely sensitive to the valency of the molecular interaction network (Li et al. 2012), the binding of SAMs to synaptic scaffold proteins creates a positive feedback loop for SAM-instructed AZ and PSD condensate formation. This can explain why SAMs expressing nonneuronal cells cocultured with neurons can rapidly induce synaptic clustering of intracellular scaffold proteins. Second, the organization of various subcompartments in presynaptic boutons and in PSDs is polarized. Binding of PSD-95 to membranes via interaction with TARPs and by its own palmitoyl groups ensures that the PSD core is always near the membrane, but the lower layers of PSD scaffold proteins such as SAPAP, Shank, and Homer are more distal to the membrane (Dosemeci et al. 2016). Similarly, the AZ is attached to presynaptic membranes, and reserve pool SV/Synapsin condensate is more distal to plasma membranes (Milovanovic & De Camilli 2017). Such polarized organization of multiple compartments on both sides of a synapse is critical for synaptic signaling and plasticity as we described above. Third, through transsynaptic SAM interactions, functional condensates such as presynaptic AZ nanoclusters and associated SNARE machineries can align with the neurotransmitter receptor nanoclusters organized by the PSD condensate (Haas et al. 2018, Han et al. 2022, Martinez-Sanchez et al. 2021, Ramsey et al. 2021). Finally, the transsynaptic SAM complexes, possibly together with synaptic extracellular matrix proteins, may form condensed molecular assemblies in the synaptic cleft via phase separation. The electron-dense molecular organization in the synaptic cleft has been observed by EM for different synapses (Cole & Reese 2023, High et al. 2015, Zuber et al. 2005). Such dense synaptic cleft molecular organization has been suggested to be a possible mechanism for synapses to encode long-term memories (Tsien 2013).

SUMMARY

A synapse is a tiny signaling compartment in the context of the whole nerve cell. A tiny synapse is further compartmentalized into multiple subcompartments to ensure directional signal flow from one neuron to another with exquisite control of timing, speed, and amplitude and dynamic

regulation of signal transmissions. Increasing evidence from the past eight years or so indicates that the organization of molecular complexes into condensed molecular networks via phase separation allows distinct subsynaptic functional compartments to form autonomously on both sides of synapses. These functional subsynaptic compartments/condensates do not contain physical barriers, but they occupy distinct subsynaptic regions and coordinate with each other to conduct neuronal signal transductions. The densities of molecular components as well as the physical sizes of subsynaptic condensates can be dynamically regulated to match with the plasticity requirements of neuronal synapses. The discovery of phase separation in organizing subsynaptic compartments/condensates is an exciting development in molecular cellular neuroscience. Many new discoveries in this area are expected to follow in the coming years. It should be noted that the field is in its infancy. Biological condensates formed via phase separation are complex condensed matters with emergent properties that do not exist in homogeneous dilute solutions. For example, molecules in biological condensates behave fundamentally differently from the same molecules in dilute solutions, but we are short on mature theories for describing molecules in biological condensates. Current theoretical models for phase-separated systems are mainly based on simple polymers, which cannot fully recapitulate the complexity of biomolecules. Additionally, technologies and methods devised for studying dilute solutions are often not suitable for investigating molecules in condensed phases. Thus, suitable techniques and methods for biological condensates are urgently needed. Deep understanding of such complex and condensed-matter systems will require collaborative efforts from scientists from multiple disciplines, including, but not limited to, neuroscience, biochemistry, biophysics, physics, and engineering.

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