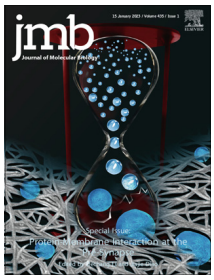

Interactions between Membraneless Condensates and Membranous Organelles at the Presynapse: A Phase Separation View of Synaptic Vesicle Cycle

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Abstract

Action potential-induced neurotransmitter release in presynaptic boutons involves coordinated actions of a large list of proteins that are associated directly or indirectly with membrane structures including synaptic vesicles and plasma membranes. These proteins are often highly abundant in different synaptic bouton sub-compartments, and they rarely act alone. Instead, these proteins interact with each other forming intricate and distinct molecular complexes. Many of these complexes form condensed clusters on membrane surfaces. This review summarizes findings in recent years showing that many of presynaptic protein complex assemblies are formed via phase separation. These protein condensates extensively interact with lipid membranes via distinct modes, forming various mesoscale structures by different mode of organizations between membraneless condensates and membranous organelles. We discuss that such mesoscale interactions could have deep implications on mobilization, exocytosis, and retrieval of synaptic vesicles.

Introduction

Neurotransmitter release is a discrete and quantal event. The quantal release theory is established based on the package of neurotransmitters into isolated synaptic vesicles (SVs) and discrete fusion between a single SV and the plasma membrane.[1–3] The number of SVs in a presynaptic bouton varies from a few dozens to hundreds of thousands depending on the species and types of synapses, with an average of a few dozens to a couple of hundreds in the mammalian central nervous system.[4–5] Not all SVs stand a chance to fuse with the plasma membrane but instead, only a minor cohort that intimately interacts with the presynaptic plasma membrane can achieve this purpose.[6–8] SVs are generally classified into three pools based on their localization within the presynaptic bouton and their functioning in neurotransmitter release.[9–10] (Figure 1) The reserve pool, which represents the largest pool of SVs (>80% of total SVs), is located most distant from the presynaptic plasma membrane and serves as a SV reservoir. The release-ready pool or readily releasable pool (RRP) refers to SVs (<5% of total SVs) that land within the active zone. These vesicles can immediately respond to stimulations within the termini and fuse with the plasma membrane. Finally, the recycling pool includes SVs that dynamically shuttle between the reserve pool and the plasma membrane, either to replenish the RRP to sustain continuous neurotransmission or to deposit endocytic SVs back to the reserve pool to maintain homeostasis of biomass. The physical localization

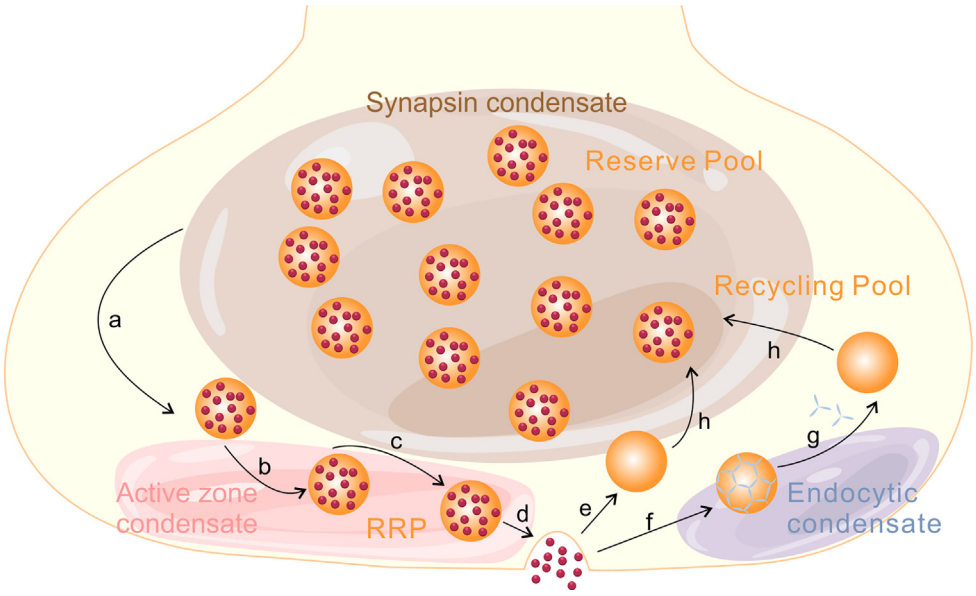

Figure 1. Phase separation view of SV cycle. The SV cycle can be roughly divided into three stages: resting state, exocytosis, and endocytosis. These processes occur in specific spaces in a presynaptic bouton: synapsin condensate, active zone condensate, and endocytic condensate, correspondingly. SVs are grouped into three pools: reserve pool, readily releasable pool (RRP), and recycling pool. Phase separation has been demonstrated to participate in the organization of all three condensates, and modulation of all the SV pools. At the resting state, synapsin condensate gather reserve pool SVs; during synaptic activity, SVs transport from reserve pool and be tethered to the active zone condensate (a). At the active zone, SVs are subject to docking (b) and priming (c) and to be release-ready. Upon Ca^{2+} influx, SV undergoes fusion with the plasma membrane (d). After fusion, multiple ways of endocytosis happen on the endocytic condensate, for example, "kiss-and-run" (e) and clathrin-mediated endocytosis (f). Further step like uncoating generates empty SV (g). Finally, the empty SV is refilled with neurotransmitter and re-enter the SV cycle (h).

instead of the molecular composition determines the identity of SVs (i.e., which pool does an SV belong to).[10] Therefore, it is hard to find biological markers to differentiate between different pools of SVs, although the association with different proteins localized at specific region may help to determine the localization and identity of SVs.[11–12]

An important pillar of modern cell biology is the discovery of lipid membrane-demarcated organelles in cells. These membrane-enclosed organelles enable compartmentalization of cells for distinct chemical reactions and biological activities. However, cells also harbour a broad variety of sub-compartments (e.g., Cajal bodies) that are not fully enclosed with membranes. How these organelles are formed and how they achieve spatiotemporal control on their molecular compositions have long remained unanswered. Extensive studies carried out in recent years start to reveal that phase separation is likely the general organization principle underlying the formation of non-membrane bound or semi-membrane bound organelles.[13–17] Typical examples include P granules,[18–20] RNA granules,[21–23] stress granules,[24–26] nucleoli,[27–29] etc. Phase separation governs the biogenesis of these subcellular condensates in responding to various cellular signals. Aberrant phase separations related to these condensates are linked to various diseases in humans.[30–32] Phase separation is a spontaneous process in which homogenously distributed macromolecules (e.g., proteins and nucleic acids) can autonomously partition into a condensed phase coexisting with a dilute phase when components reach supersaturation.[33–35] Large molecular assemblies formed via phase separation are usually referred to as biomolecular/biological condensates, membraneless compartments/organelles, or liquid droplets. For simplicity, we use condensates throughout this review. The most important feature of these condensates is that, without a delimiting membrane, highly concentrated molecules or molecular assemblies can be stably maintained within a condensed phase despite the huge diffusion potential created by the sharp concentration gradient between the condensate and the surrounding solution, and yet molecules between the two phases can freely exchange. Both external environmental conditions (pH, temperature, ionic strength, crowding reagent, etc.) and intrinsic signalling events (post-translational modification, redox state, ligand activation, chaperon, etc.) can

regulate formation and organization of biomolecular condensates.[36–40]

Phase separation has profound impacts on biological activities of condensates at multiple scales.[41] The concentration of molecules within a biological condensate can be tens to thousands of folds higher than the surrounding milieu. At the microscopic level, molecules in the condensed phase often have distinct properties from their surroundings. For example, phase separation can alter the catalytic activity of enzymes,[42–43] the dwell time of constituents,[44] and the folding of proteins and enzymes.[29] Additionally, membraneless condensates can selectively concentrate or exclude molecules within a defined foci.[45] The size of membraneless condensates in cells formed via phase separation is normally at micron scale. At the subcellular scale, phase separation governs the formation, maintenance, location, and oriented organization of mesoscale membraneless condensates.[15–16] It is well known that different membrane organelles can interact with each other at various membrane contact sites.[46–47] Similarly, membraneless condensates and membrane organelles are also able to interact with each other.[48] Thus, the appreciation of membraneless condensates in cells, in a way analogous to the discovery of membrane organelles, represents another major advancement in cell biology. However, the field of membraneless condensate research is still in its infancy.

Nerve cells share many common features found in typical mammalian cells. A plethora of membraneless condensates were initially identified in the nervous system. For instance, a star molecule in the phase separation field – Fused in sarcoma (FUS), is one of the very first proteins shown to undergo phase separation both in vitro and in vivo.[21–22,49] Dysregulations of these condensates are linked to neurological diseases.[31,50] Those mesoscale interactions that are shared among all cells will not be discussed here, and we recommend interested readers to several recent reviews.[48,51–53] Neuronal signal is mainly transduced at chemical synapses, where electric signals (action potentials) are converted into chemical signals (neurotransmitters) at the presynapses of signal transmitting nerve cells and trigger electric responses at the postsynapses of signal receiving nerve cells. Many phase separation-mediated condensates have been identified within synapse, which is composed of a presynaptic bouton and the perfectly aligned postsynaptic dendritic spine protrusion. These synaptic membraneless condensates have been linked to the structural organization and synaptic functions of nerve cells.[54–59] Different from most of the membraneless condensates in cytosol, condensates in synaptic regions are usually associated with membranes – plasma membrane or SVs. In this review, we summarize recent studies that show phase separation driven molecular organizations within presynaptic termini with a focus on how distinct condensates function in SV cycling.

Resting state

Three models of SV clustering by synapsin

The reserve pool SV cluster is one of the most conspicuous features of a presynaptic bouton. Different from other organelles, SVs tend to gather, leaving a clear boundary segregating them from the cytoplasm. A long pending question is how these SVs keep glued together yet remain highly mobile.[60–62] Under stimulation, SVs are transported to the plasma membrane to facilitate neurotransmission. After fusion, membranes are recycled through endocytosis. The endocytic vesicles will gradually diffuse in and replenish the reserve pool of SVs.[63–64] In addition, SVs are captured to exchange between multiple synapses.[65–66] (Figure 1).

Three models have been proposed for the clustering of SVs, all of which rely on a key protein – synapsin. Synapsin is the first protein discovered to associate with SVs, and accounts for 5 $\sim$ 10% of total proteins associated with SVs.[12,67–69] Either injection of synapsin antibody or knockout of synapsin resulted in drastic depletion of reserve pool SVs.[70–72] But how does synapsin achieve this goal has been a highly debated topic.[73–74] The first model of SV clustering is based on actin cytoskeleton. Synapsin directly interacts with actin and promotes actin bundling.[75–76] Linking SVs to the cytoskeleton seems a good way to gather SVs.[77] However, the precise localization and function of actin filaments in a presynaptic bouton is highly debatable.[5,78–79] And more importantly, the large reserve pool is retained even after actin depolymerization,[80–81] suggesting that actin-independent mechanisms are involved in the maintenance of reserve pool SVs. The second model of SV clustering suggests that SVs are crosslinked with each other via connectors including synapsin through direct interaction.[5,82–84] But there are only a few numbers (2 $\sim$ 4) of connectors visible under electron microscopy (EM).[5] An independent mass spectrometry study with purified SVs reported that $\sim$ 8 copies of synapsin are associated with one SV.[12] However, this number is far below the concentration of synapsin measured by synaptic bouton protein purification, where over 23,000 copies of synapsin were counted per synapse.[68] Assuming an average level of 200 SVs per bouton,[4] each vesicle would be surrounded by > 100 synapsin molecules instead of just a few observed experimentally. Thus, not all synapsins are tethered with SVs.

A recent study brings forward the third model referred to as the phase separation model. Recombinantly purified synapsin protein could undergo phase separation in the presence of other binding partners or crowding reagents.[85] Further mapping experiments revealed that the intrinsically disordered D domain, which contains multiple proline-rich motifs (PRMs), is responsible for synapsin phase separation. Several SH3 domain-containing proteins, such as intersectin (ITSN) and GRB2, were incorporated into synapsin condensates via direct interaction with the D domain. Importantly, the synapsin condensate was able to capture and concentrate negatively charged small unilamellar vesicles (SUVs) but not SUVs prepared from neutral lipids, reminiscent of the clustered reserve pool SVs in a presynaptic bouton.[85] The synapsin-mediated phase separation model reconciles divergences in the first two models and furnishes a new paradigm in understanding SV organization.[85–86] This model bypasses the requirement of actin filaments but does not contradict with the regulatory role of actin. Furthermore, synapsin self-association contributes to its condensation and resolves the apparent dilemma of the unusually high stochiometric ratio of synapsin over SVs. Probably more importantly, both synapsin and vesicles in condensates remain highly mobile, which enables SV mobilization in response to stimulation. Synapsin can dissociate from SVs upon simulation and this process is reversable.[87] In a minimal reconstitution system in vitro, Ca^{2+}/CaM activated CaMKII causes dissolution of synapsin/SUV condensates, which mimics the action potential triggered activation of CaMKII and subsequently the synapsin phosphorylation-induced SV dispersion in vivo.[77,88] Synapsin domain D was found to be specifically phosphorylated by CaMKII, which then disrupted the interaction of synapsin with SVs and released SVs from the reserve pool for subsequent steps in the fusion process.[89–90] An antibody injection assay performed in living lamprey giant reticulospinal synapses provided further evidence to support the phase separation model for SV clustering. Injection of an antibody against synapsin D domain can dissolve reserve pool vesicles in neurons maintained at the resting state.[86] But surprisingly, in the in vitro reconstitution system, synapsin D antibody promoted synapsin phase separation in stark contrast to the condensate dispersion observed in living neurons.[86] Currently, there lacks an explanation for the contradictory observations made in in vitro and in vivo.[74] Nonetheless, the involvement of synapsin phase separation in SV clustering is further supported by observations showing that the addition of the ITSN SH3A domain, a synapsin D domain binding protein, disrupted synapsin condensation in vitro and caused SV dispersion in vivo,[86] and injection of an antibody against synapsin E domain in the same model system could also cause vesicle dispersion under stimulation state.[70]

Some unresolved issues on synapsin phase separation-mediated SV clustering and regulation

The in vitro reconstitution experiments have demonstrated that CaMKII could regulate synapsin-mediated SV clustering, but the study used a very high enzyme to substrate ratio (0.9 or 9 μM CaMKII: 1 μM synapsin) in the assay.[85] In addition, synapsin was probably the first identified protein substrate for PKA in the central nervous system, and it was named as protein I.[67,91] The PKA phosphorylation site (site 1 at Ser 9) located in domain A of synapsin is shared among all isoforms, whereas CaMKII phosphorylation sites 2 (Ser 566) and 3 (Ser 603) located in domain D (or domain J for synapsin III) are only present in synapsin I and III, but not in isoform II.[90] This difference in the phosphorylation site conservation suggests PKA-mediated phosphorylation may have a broader effect on synapsin regulation.[92] In glutamatergic synapses, double knockout of synapsin I and II or triple knockout of synapsin I, II, and III does not affect the single action potential-triggered neurotransmission but accelerates synaptic depression under successive stimulations,[71–72] which is in agreement with the notion that synapsin specifically controls the reserve pool maintenance. But this depression can only be rescued by the expression of synapsin II but not synapsin I.[93] Oddly, synapsin II does not seem to be regulated by CaMKII. Whether CaMKII-mediated phosphorylation of synapsin is the mechanism underlying synapsin condensate dissolving under physiological conditions and whether PKA phosphorylation is also involved in synapsin condensate regulation need to be addressed in the future.

Synapsin extensively interacts with other proteins, among which α-synuclein stands out due to its pathological roles in Parkinson's disease.[94–96] Injection of an antibody against pan-synucleins resulted in the loss of both reserve and docked pools of SVs,[97] suggesting an indispensable role of synucleins in SV organization. Consistently, synuclein knockdown severely depleted reserve pool SVs.[98] These studies suggest a cooperative role between synuclein and synapsin in SV clustering. Direct interaction between synapsin and synuclein was verified recently and was shown to be essential for α-synuclein to facilitate SV clustering.[99] It was further demonstrated that α-synuclein could undergo phase separation on its own under certain extreme conditions,[100] but whether this protein can phase separate under physiological conditions is under debate.[101] Nonetheless, it was

observed that α-synuclein could partition into and in return modulate synapsin/SV condensation.[101] Synuclein was also indicated to modulate SV clustering independent of synapsin. Triple knockout of α-, β, γ-synucleins impaired the assembly of SNARE complex, via direct interaction with synaptobrevin.[102–103] Native and recombinant α-synuclein can induce synaptobrevin-containing liposome clustering.[104] Understanding how synuclein cooperates with synapsin in SV organization and whether it is involved in docking and recycling of SVs, will certainly be interesting directions for future investigation.

SV biogenesis and trafficking

In addition to SV clustering, synapsin may also be involved in SV biogenesis. A recent heterologous cell-based reconstitution assay offered insights into the interplay between SV biogenesis and phase separation. Synaptophysin, an abundant SV resident protein,[12,105–106] formed liquid droplet-like structures when co-expressed with synapsin in COS7 cells. In contrast, expression of either protein alone could not form such droplet-like structures,[107] indicating that synaptophysin, likely via direct binding, can modulate synapsin phase separation. It was proposed that the negatively charged residues in the cytoplasmic tail of synaptophysin might interact with the positively charged residues in domain D of synapsin.[107] The C-terminal tail of synaptophysin also contains ten short repeats with a consensus sequence motif of "Y-G-P/Q-Q-G".[108] A recent study demonstrated that substitution of tyrosine residues in the repeating sequence with serine blocked synapsin/synaptophysin phase separation, suggesting phase separation is governed by cation-π interactions.[109] Correlative light-electron microscopy study demonstrated that synapsin/synaptophysin condensates assembled in heterologous cells are filled with clustered small vesicles.[107] These vesicles are reminiscent of reserve pool SVs observed in the presynaptic bouton in the following aspects. First, vesicles gathered in heterologous cells share comparable sizes with the bona fide SVs, and numerous vesicles are tightly coupled together leaving a sharp boundary between the dense vesicle clusters and the bulk cytoplasm. In contrast, overexpression of synaptophysin alone, however, only led to small and loosely clustered vesicles.[110–111] Second, the tight clusters of small vesicles are quite mobile, as indicated by the random distribution of endocytic tracer-marked vesicles within the synapsin/synaptophysin condensates. These vesicle containing condensates can fuse with each other and can be dispersed by active CaMKII. Therefore, it appears that synaptophysin and synapsin together may serve as a minimal set of proteins required for SV biogenesis and maintenance.[107] However, unlike the severe defects in SV clustering observed in synapsin knockout mice, synaptophysin knockout neither led to obvious alterations in SV clustering nor caused visible ultrastructural changes of synapses.[112] It is possible that certain compensatory mechanism may play a redundant role in the synaptophysin knockout animals.

Another related question is how SVs are transported to the nerve terminal after biogenesis. Members of Rab-family small GTPases control vesicle trafficking and are widely recognized as vesicle markers.[113] Recently, it was reported that Rab6, via interaction with ELKS, plays critical roles in the trafficking of SVs to synaptic boutons.[114] Biochemically, both ELKS1 and ELKS2 share similar Rab6 binding property. However, only ELKS1, but not ELKS2, is positioned to capture SVs at the synapse. Stimulated emission depletion (STED) imaging study revealed that ELKS2 is preferentially restricted at the active zone, whereas ELKS1 is widely spread throughout the entire nerve terminal and is thus positioned to capture trafficking SVs.[114] ELKS1 and ELKS2 share 71% identity and 83% similarity in protein sequences (EMBOSS Stretcher), and share key biochemical properties.[115–116] It will be interesting to figure out what determines different localizations of the two ELKS proteins and subsequently their different functional roles in SV trafficking.

SV exocytosis

Upon the arrival of an action potential in the presynaptic terminal, SV releases neurotransmitters through exocytosis. Fusion of SVs with the presynaptic plasma membrane occurs within a region called active zone and is executed by SNARE proteins. SV undergoes sequential steps including tethering, docking, and priming before it becomes competent for fusion, and these processes are sophisticatedly regulated by many proteins. Decades of studies have identified many specific protein–protein and protein-lipid interactions involved in SV exocytosis. Recent discoveries that phase separation may underlie the organization of some of these proteins or protein complexes provide new insights to better understand numerous experimental observations accumulated in this well studied topic (Figure 2(A)).

Active zone protein phase separation and SV mobilization

Active zone is situated right beneath the presynaptic plasma membrane where SV fusion occurs.[117–118] Structurally, active zone is characterized by the presence of electron-dense proteinaceous materials – also known as dense projections under EM.[119] Morphologies of active zones vary among different types of synapses.[117,120] Functionally, active zones participate in the entire process of SV mobilization, which

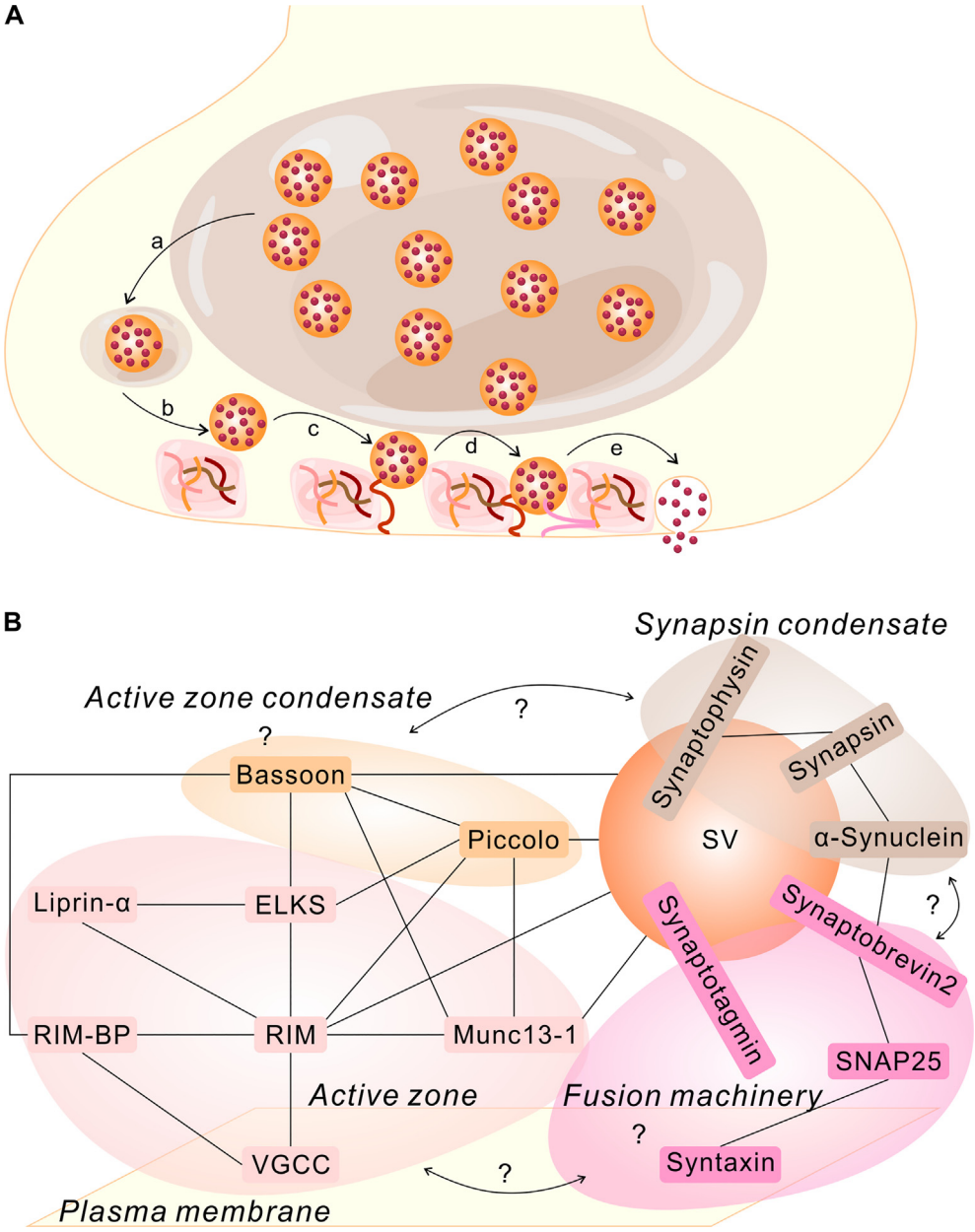

Figure 2. SV exocytosis. **A**. Graphic illustration of phase separation mediated SV exocytosis. (a) under stimulation, synapsin condensate is partially dissolved and SVs are liberated from the reserve pool. (b) active zone condensate can capture and tether SVs. (c) additional proteins are involved in docking SVs closer to the plasma membrane. (d) other components prime SVs in a release-ready state. (e) Ca^{2+} influx triggers SV fusion with the plasma membrane. **B**. Key molecules and their interactions that are critical in SV exocytosis. Each line indicates one pair of interaction. Multiple active zone components (like RIM, Munc13, etc.) interact with each other and with SV protein or membranes to form phase separated condensates. Whether Bassoon and Piccolo may form condensates and, if yes, how such condensates may interact with the RIM-containing condensates are not known. Whether the SNARE fusion machinery may phase separate is to be tested. How the SNARE fusion machinery may interact with the synapsin condensate is unknown.

includes tethering, docking, priming, and fusion of SVs once they are released from the reserve pool (Figure 2(A)). In addition, active zones determine the positioning and enrichment of voltage-gated calcium channels (VGCCs), mediate the trans-synaptic alignment with postsynaptic receptors, and participate in the establishment of presynaptic plasticity.[117,121–122] Active zone can be biochemically purified as a detergent insoluble fraction.[68] Active zones from both vertebrates and invertebrates contain five major scaffold proteins (Rab3-interacting molecule (RIM), RIM-binding protein

(RIM-BP), ELKS, Liprin-α, and Munc13) intertwined with each other.[117] Active zones from vertebrates contain two additional gigantic proteins: Bassoon and Piccolo.[117,123] These seven proteins likely form the basic architecture of the active zone to support essentially all known activities occurring within this confined space. Besides, many other proteins, such as the SNARE fusion machinery and cofactors, ion channels, synaptic adhesion molecules and cytoskeletons are also known to locate within or in peripheral to active zones,[118,121,124] forming a highly intricate molecular interaction network right beneath the plasma membrane (Figure 2(B)).[117,125]

Decades of genetic studies have elucidated key roles of individual scaffold proteins in controlling SV exocytosis.[117–118] For example, RIM, RIM-BP, ELKS, and Liprin-α are involved in SV tethering and docking, RIM and Munc13 are critical for SV priming. It has always been curious that copy numbers of these key AZ scaffolding molecules are not very high, ranging from a few dozens to thousands per bouton (∼0.1–10 μM).[68] A strategy to counter their low protein concentration is by redundancy and compensation. All these scaffold proteins mentioned above contain multiple isoforms and share certain structural similarities (e.g., all are multimodular proteins and often share similar protein or lipid binding modules). For example, RIM has four genes (RIM1, 2, 3, 4) and each has three transcripts (α, β, γ).[126] RIM, Munc13, and Piccolo share C2 domains,ELKS, Liprin-α, Bassoon, and Piccolo share coiled coil domains,RIM and Piccolo share PDZ domains,and RIM, Bassoon, and Piccolo share zinc finger (ZF) domains and PRMs. Consequently, multiple proteins work together and function redundantly in the active zone assembly. The complexity of active zone machinery has made it challenging to dissect mechanisms underlying SV exocytosis and requires simultaneous manipulation of multiple genes to determine their functions. For instance, simultaneous removal of all brain isoforms of RIM and RIM-BP in mice totally abolished the dense projection structure, resulting in deficient SV docking and fusion.[127] Similarly, simultaneous knockout of RIMs and ELKSs, but not deletion of the individual families, led to the complete loss of active zones and synaptic transmissions in mice.[128]

Mechanistically, how multiple proteins may work together? Phase separation-mediated active zone formation provides a potential explanation. In a pioneering study, mixtures of recombinantly purified RIM and RIM-BP proteins were shown to undergo phase separation driven by multivalent interactions between the two proteins.[129] The intrinsically disordered sequences of RIM also contributed to phase separation and lowered the threshold of condensation.[129] This in vitro reconstitution study provided a new biochemical framework to understand active zone organization. Interestingly, an earlier stochastic optical reconstruction microscopy (STORM) imaging study uncovered that RIM forms nanoclusters in presynaptic bouton,[130] consistent with RIM condensation observed in vitro. Similarly, RIM-BP, ELKS, Liprin-α, Munc13, Bassoon, and Piccolo have also been shown to form nanoclusters within a narrow space of < 100 nm away from the presynaptic plasma membrane.[68,131–133] It remains to be tested whether phase separation is also involved in their confined localization. If it is true, phase separation might be a general organizing principle in the active zone.

RIM/RIM-BP phase separation does not require lipid membrane, but membranes do involve in their condensation. The time course between the arrival of an action potential and the occurrence of vesicle fusion is less than one millisecond.[134–135] Two molecular events govern this ultra-fast neurotransmission: 1) the close proximity between Ca^{2+} and its sensor – synaptotagmin residing in SVs,2) the clustering of multiple VGCCs.[136] Both events are closely linked with the distribution of VGCCs on the plasma membrane. The intracellular C-terminal tail (CT) of VGCC can directly interact with RIM and RIM-BP. Tethering of purified VGCC-CT on supported lipid bilayer (SLB) allowed the RIM/RIM-BP mixture to phase separate on the membrane surface, analogous to the assembly of active zone scaffolds beneath the presynaptic plasma membrane.[129] Importantly, membrane-attached RIM/RIM-BP condensates induced the formation of VGCC-CT clusters on the SLB. Thus, it is plausible that phase separation of RIM/RIM-BP can cluster VGCCs in the active zone and couple the clustered channels with the SNARE fusion machinery possibly via other molecules such as Munc13 (Figure 2(B)), thereby controlling amplitudes and kinetics of neurotransmitter releases induced by action potentials.

In addition to engaging plasma membranes, RIM/RIM-BP condensates can also directly interact with SVs and such interaction requires negatively charged lipids on vesicles.[115] When in vitro reconstituted RIM/RIM-BP condensates were incubated with SVs purified from the rat brain, it was striking to observe the coating of SVs on the surface of protein condensates,[115] a phenomenon that does not rely on SV proteins such as Rab3, which is known to bind RIM.[137] This in vitro observation may provide an explanation for why the number of docked vesicles in a synaptic bouton is linearly proportional to the surface area of its active zone.[138–139] As discussed above, SVs can coacervate with synapsin condensates. Both populations of SVs exist within a presynaptic bouton (Figure 2(A)), and it is therefore puzzling how the RIM/RIM-BP condensate and the synapsin condensate co-exist but segregate from each other in a confined tiny presynaptic bouton. Remarkably, the synapsin/ITSN condensates encapsulate the SUV-coated RIM/RIM-BP condensates when the two phase separated-condensates were mixed.[115] This minimal reconstitution system nicely recapitulates the protein/SV

organization in presynaptic boutons where reserve pool SVs are clustered by synapsin and docked SVs are tethered on to the surface of active zone (Figure 2(A)). To further generate an in vitro system that can recapitulate the two pools of SVs in a presynaptic bouton, VGCC-CTs were tethered on giant unilamellar vesicles (GUVs). RIM/RIM-BP condensates induced the clustering of VGCC-CTs on the GUV membrane surface on one face and coated SUVs on the other face. SUV/synapsin condensates in solution surround the RIM/RIM-BP condensates attached to the GUV (Figure 1).[115] The observation of SV coating on RIM/RIM-BP condensates raised a question of how SVs may penetrate the active zone condensate for access to the presynaptic plasma membrane? In real synapses, multiple dense projections with $\sim$ 50 nm in diameter are attached to the plasma membrane forming grid-like structures.[140–141] Thus, gaps between dense projections can accommodate SVs that are $\sim$ 40 nm in diameter. A recent study showed that biomolecules may serve as Pickering agents to control the size of condensates.[142] In this case, it will be interesting to test whether SVs, alone or together with other proteins, may function as a Pickering agent to shape the active zone in a grid-like structure on the presynaptic plasma membrane surface, and in return gain access to the membrane (Figure 2(A)). For readers interested in the size control of phase separated-condensates in vitro and whether biophysical properties are transferable between condensates with large size differences, we refer them to a recent review.[41]

Apart from RIM and RIM-BP, other active zone proteins were also reported to undergo phase separation. Among them, ELKS directly interacts and phase separates with RIM.[115] Incorporation of ELKS into the RIM/RIM-BP condensates does not affect the distribution of SUVs, which is in agreement with genetic studies that simultaneous knockout of RIM and RIM-BP or RIM and ELKS led to similar consequences in terms of active zone structural organization and SV behaviours.[127–128] More recently, two other studies independently reported that ELKS alone can phase separate.[143–144] Both studies concluded that intrinsically disordered regions within ELKS drive phase separation, although the exact regions mapped for phase separation are completely different from the two studies. In a more recent genetic study in mice with simultaneous knockout of RIM and ELKS, re-expression of RIM nearly restored the localization of all other proteins on the plasma membrane as well as a partial ($\sim$50%) SV docking, whereas re-expression of ELKS alone barely rescued these phenotypes at all.[145] These observations suggest that RIM plays essential and major roles in active zone organization but ELKS is also indispensable for SV mobilization.

Another active zone scaffold protein Liprin-α, which is essential for the recruitment of RIM and Munc13,[146–148] was also demonstrated to undergo phase separation.[143–144,149] When expressed in HEK293T cells, mVenus-Liprin-α3 formed spherical droplets after PKC-mediated phosphorylation on Ser760, indicating a highly specific and regulated process. The integrity of Liprin-α3 protein is required for condensation, as any shorter fragments failed to form droplets.[149] Molecular mechanisms driving Liprin-α phase separation differ between functional orthologs in humans and C. elegans.[143,149] In the reconstituted heterologous cell system, when RIM and Munc13 were co-expressed with Liprin-α3, all three proteins were colocalized in the same condensates. Upon contact with the plasma membrane, these condensates became flatter, reminiscent of the active zone attachment on the presynaptic plasma membrane.[149] In Liprin-α2 and -α3 double knockout mice, synaptic levels of RIM and Munc13 were decreased even though the remaining proteins were properly localized. Consequently, the amount of docked SVs was significantly reduced.[149] Similarly, in C. elegans, the SYD-2 (worm ortholog of mammalian Liprin-α) and ELKS phase separation deficient mutations led to significantly diminished localization of UNC-10 (worm ortholog of mammalian RIM) and UNC-13 (worm ortholog of mammalian Munc13) and disrupted SV docking in presynaptic termini.[143] Together, these results suggest that Liprin-α, RIM, and Munc13 are all involved in the assembly of active zone and in the tethering and docking of SVs.

Munc13 is a priming factor essential for SV exocytosis.[150–151] It was discovered to function as the core molecule to form a supramolecular assembly that defines the neurotransmitter release site by STORM microscopy.[152] Immunogold staining also uncovered that Munc13 forms nanoclusters and its distribution pattern and relative distance to VGCCs determine the synaptic strength.[153] It is possible that Munc13 might also undergo phase separation. Full-length Munc13 protein can be divided into two parts- N and C segments, with the N segment consisting of a homodimerized C2A domain and a long linker, and the C segment consisting of a long MUN domain flanked by multiple membrane binding domains (C1 and C2B at the N terminal, C2C at the C terminal of MUN). A large body of reconstitution assays focused on the C segment – Munc13-C (C1-C2B-MUN-C2C), since it is directly involved in regulating SNARE complex assembly and bridging SVs to the plasma membrane. In vitro purified Munc13-C protein was shown to cluster multiple SUVs in solution.[154–156] When tethered on SLBs, Munc13-C forms nanoclusters that can capture and retain SUVs.[157] A very recent cryo-EM study reveals that Munc13-C can adopt trimeric open conformation or hexameric closed conformation,[158] revealing that the molecule has the capacity to form multivalent network structures. The N part of Munc13 is grossly termed as an “autoinhibitory” segment, which is released by

RIM binding.[159] This N part is generally left out in biochemical studies due to challenges in its purification. However, even the constitutively open form of Munc13 still requires RIM binding for proper vesicle docking and priming,[160] suggesting that RIM plays additional roles apart from the release of autoinhibition. Similar to Munc13, RIM can also be divided into N and C segments, with the N part consisting of a ZF domain responsible for Rab3 and Munc13 binding and a long linker, and the C part consisting of a PDZ domain and a PRM for binding to VGCCs and RIM-BPs respectively, and two C2 domains for interaction with Liprin-α and the plasma membrane.[161] The integrity of RIM is essential for its function as the expression of any truncated fragments in RIM knockout neurons cannot rescue the proper distribution of other proteins, nor the SV docking and fusion.[145,162] Interestingly, fusion of the ZF domain of RIM to VGCCs simultaneously restored the binding and recruitment of Munc13 and subsequently the docking, priming and fusion of SVs.[145] At the first glance, this artificial VGCC-ZF fusion chimera appears to bypass the requirement of its C segment and the need of RIM phase separation. But in living synapses, neurotransmitter release is undoubtedly much more complicated in terms of robustness, intricacy of regulation, time and speed of release, etc. Assembly of the active zone molecular network via phase separation provides multiple ways (e.g., multiple proteins act redundantly) to concentrate Munc13 near both VGCCs and the SNARE fusion machinery. Additionally, different components in the active zone condensate may respond to different regulatory signals to tune kinetics and amplitudes of neurotransmitter release (Figure 2(B)). It is certain that much will need to be investigated in the future to understand function of phase separation in active zone assembly and organization.

Fusion machinery assembly

Once primed to the release-ready state, SVs are subject to SNARE-mediated membrane fusion in response to Ca^{2+} influx. Discussions above have established that phase separation of active zone scaffold proteins may contribute to SV tethering, docking, and priming. How about fusion? In vitro reconstitution studies have demonstrated that the SNARE complex is sufficient to mediate membrane fusion without any other cofactors,[163] but the time duration required for this process is way too long.[164] Recent reconstitution assays by including Munc13, Munc18, synaptotagmin, complexin, NSF, and α-SNAP to the SNARE complex have vastly accelerated the fusion speed to millisecond level.[165–166] Could protein phase separation further help to speed up the fusion event? SNARE proteins are among the top abundant proteins in a presynaptic bouton, with > 100 μM in each bouton.[68] This concentration is much higher than the number of SNAREs needed for fusion events.[167] One might hypothesize that if SNAREs and regulators are enriched via phase separation in a way analogous to synapsin-mediated SV clustering, the fusion process could be further speeded up to physiologically relevant rates. STED fluorescence microscopy imaging of PC12 cells revealed that syntaxin forms clusters with a diameter of 50 ~ 60 nm on the plasma membrane.[168] Further imaging analysis and simulation studies proposed that protein self-association may counteract the potential steric repulsions induced by crowding effect and thus to maintain the size of the cluster to accommodate ~ 75 copies of syntaxin.[168] Similar cluster distribution of syntaxin was later observed by an independent study.[169] This study further illustrated that phosphatidylinositol-4, 5-biphosphate (PIP2) lipids arrange into microdomains with a diameter ~ 70 nm on the plasma membrane. Removal of PIP2 from the membrane by phosphatase decreased syntaxin clustering in PC12 cells.[169–170] In vitro GUV reconstitution experiment also demonstrated the necessity of PIP2 in syntaxin segregation.[169] Recent STORM imaging study uncovered that Munc13 nanocluster is essential for the recruitment of syntaxin nanoclusters to the active zone.[152] PIP2 binding and SNARE assembly further decrease syntaxin mobility and thus prevent it from dissociating from the nanoclusters.[171] Similarly, SNAP25, via direct binding to syntaxin, also forms nanoclusters on the plasma membrane.[172–173] Synaptobrevin and synaptophysin are among the most abundant proteins on SVs, with ~ 70 and 32 copies respectively per vesicle.[12] Synaptophysin assembles into a hexamer that binds to six synaptobrevin dimers, resulting in a large ring architecture.[174] One would predict that the synaptobrevin and synaptophysin complex may also form condensates if their unstructured regions are included.

The Ca^{2+} sensor – synaptotagmin, autonomously oligomerizes to form ring-like structure with a few dozens of molecules on membranes and in solution.[175–176] Formation of the ring-like oligomeric structure is likely a general feature for all synaptotagmins as well as other C2 domain containing proteins.[177] Formation of the ring-like structure is beneficial to clamp the SNARE assembly and thus to prevent the premature fusion of SVs with the presynaptic membrane.[178–179] A common feature of SNAREs and synaptotagmins is their ability to bind membranes – either via a transmembrane domain or via lipidation. Both proteins exhibit clustered patterns on membranes, implying that phase separation of the SNARE/synaptotagmin complex might occur on the membrane. The helical or disordered regions flanking the core domains of these proteins may promote phase separation of the SNARE/synaptotagmin complex.

How do SVs move from reserve pool to the active zone?

SVs docked on the active zone need to be continuously replenished in active synapses, and

these vesicles come from the reserve pool. Vesicle replenishment should be accurately coupled to SV fusion events. Therefore, SVs likely move from the reserve pool to the active zone by active transport instead of free diffusion. Bassoon and Piccolo may be the ideal vesicle carriers. Both proteins are among the largest proteins (Bassoon contains > 3900 aa, and Piccolo contains > 5000 aa) found in the human proteome. Structural analysis indicates that both proteins contain only a few of small structured domains separated by long disordered regions. 3D STORM imaging experiments revealed that both molecules adopt stretched conformation, with their C termini pointing towards the plasma membrane and N termini towards the bouton interior.[180] They can extend as long as ~ 100 nm, a length that is sufficient to connect the gap between the reserve pool and active zone condensates. Thus, Bassoon and Piccolo may play certain roles in guiding directional movements of SVs from the reserve pool to the active zone. In line with this hypothesis, knockout of Bassoon in mice prevented SV replenishment necessary for continuous fusion event, but the basal transmission remained intact.[181–182] Similarly, knockdown of Piccolo in rat neurons impaired SV transfer to the active zone but had no impact on the basal synaptic release.[183] These findings suggest that both Bassoon and Piccolo actively participate in SV mobilization and are crucial for the maintenance of SV recycling. Mechanistically, Bassoon and Piccolo interact with active zone scaffolds, including RIM, RIM-BP, ELKS, and Munc13, mainly via their C terminal domains.[123,184–185] Piccolo has also been implicated in CaMKII-dependent synapsin regulation and F-actin polymerization via binding to several cytoskeleton associated proteins using its N terminal regions.[123,186–187] It will be interesting to dissect how Piccolo and Bassoon regulate directional SV movements in synaptic boutons and whether such events involve phase separation.

Endocytosis and SV recycling

Endocytosis follows SV exocytosis to retrieve proteins and membranes from the plasma membrane and to regenerate new SVs to sustain successive neurotransmission.[6,188–189] There are a myriad of ways to mediate endocytosis, including clathrin-mediated endocytosis (CME), “kiss-and-run”, activity-dependent bulk endocytosis, and ultrafast endocytosis, etc.[190–191] (Figure 3(A)) The recycled SVs either participate in further exocytosis or refill the reserve pool, and interference with the recycling pathway can cause depletion of SVs.[192] Among the proposed endocytic pathways, CME has been increasingly appreciated to dominate the vesicle retrieval.[193–194] CME is orchestrated by dozens of proteins in a sequential manner. The entire process can be functionally defined into five steps. First, nucleation: a group of molecules including FCH domain only (FCHo) proteins, EGFR pathway substrate 15 (Eps15), and ITSN, etc determine the membrane invagination site via direct membrane binding and mutual protein interactions. Second, cargo selection: adaptor protein 2 (AP2) complex is recruited by nucleation factors and cargos. Third, clathrin coat assembly: clathrin directly interacts with AP2 and co-assembles into a membrane coat via polymerization, forming clathrin-coated pits (CCPs). Fourth, scission: Bin/amphiphysin/Rvs (BAR) domain-containing proteins like amphiphysin and endophilin sculpt membrane curvature at CCPs, and dynamin acts as a GTPase to pinch off CCPs from the plasma membrane. Fifth, uncoating: ATPase heat shock cognate 70 (HSC70) and its cofactor Auxilin disassemble the clathrin coat and liberate clathrin-free vesicles.[195–197] Perturbation to any key player along the pathway will arrest endocytosis at the corresponding step without affecting its upstream processes.[195] SV endocytosis obeys the basic principles of general endocytosis except for its faster kinetics.[188,197] Proteins participate in general endocytosis are also enriched at the presynaptic bouton suggesting these two types of endocytosis might share a similar mechanism.[188]

Eps15/FCHo phase separation initiates CME

Prior to the assembly of clathrin coat, there is an initiation step that determines where CME occurs.[198–200] FCHo1/2 are directly recruited by PIP2 lipid enriched at the endocytic sites, forming nanoclusters on the plasma membrane. FCHo1/2 further recruits Eps15 and ITSN (Figure 3(B)) via direct interaction before the arrival of downstream players AP2 and clathin.[199] Disturbance of these initiation proteins would perturb the CCP formation and SV recycling.[199,201–203] Similarly, Syp1, the yeast ortholog of mammalian FCHo, and Ede1, the yeast ortholog of mammalian Eps15, were discovered to initiate early recruitment of CME.[204] ITSN was demonstrated to co-condensate with synapsin,[85] but it is unknown whether itself can phase separate and contribute to CME. It was recently shown that the other two initiator proteins, FCHo1/2 and Eps15 (or its yeast ortholog Ede1), could undergo phase separation on their own in the presence of crowding reagent,[205–206] and their threshold concentration for condensation was lowered upon mixture, although still above the endogenous level (the endogenous level for these two proteins in Hela cells are in nM range concentration.[207] Since binding valency is a key determinant for phase separation,[208] one would predict that the inclusion of ITSN may further lower the threshold concentration for FCHo1/Eps15 condensation to physiologically relevant level. Phase separation of FCHo1 and Eps15-containing endocytosis initiation complex may partially explain why so many initiators are redundantly required.[200] When FCHo1 or Eps15 was individually tethered to PIP2-

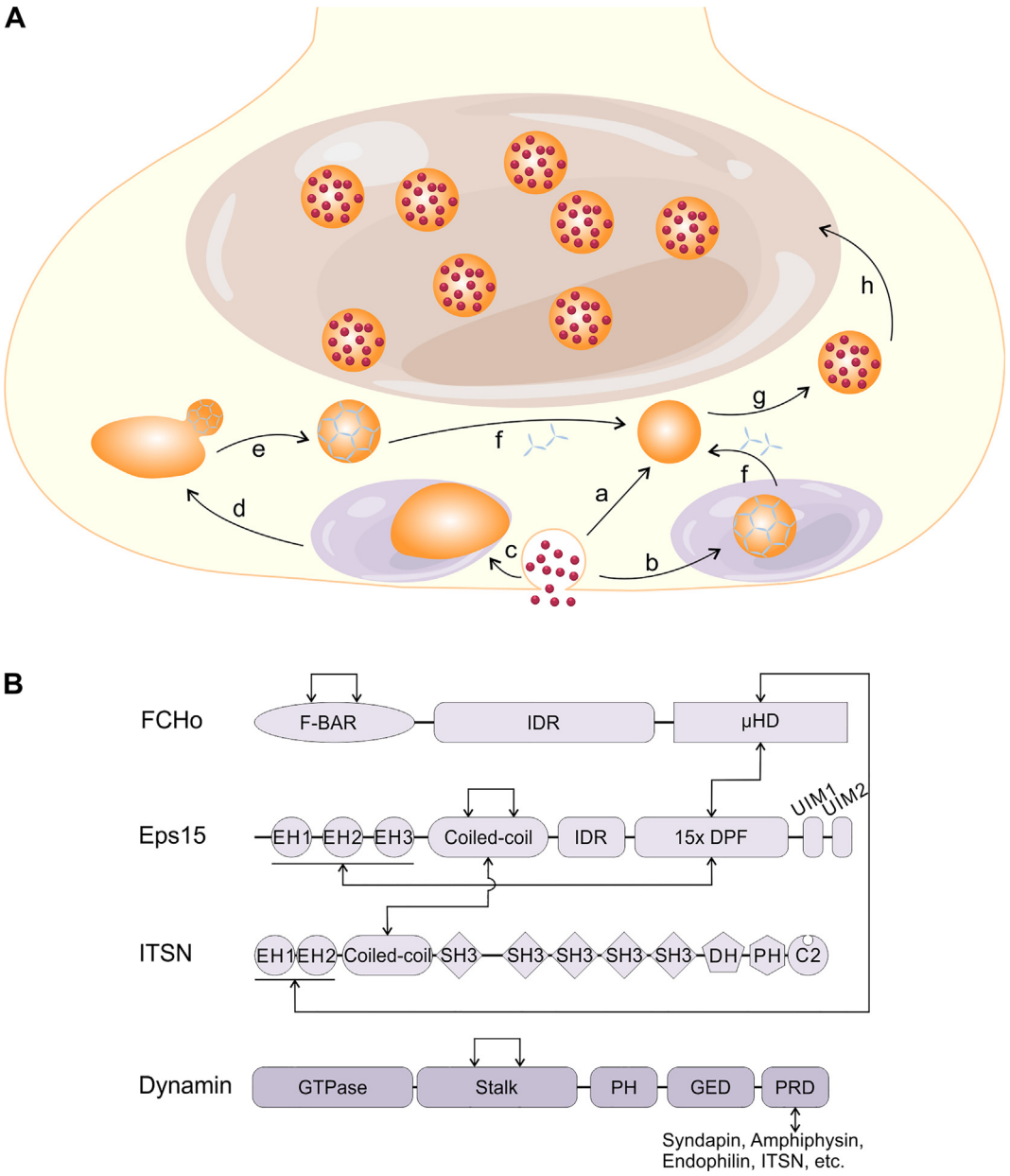

Figure 3. SV endocytosis. **A**. Illustration of involvement of phase separation in SV endocytosis. (a) "kiss-and-run" directly regenerates empty SV from the plasma membrane. (b) CME. The initiation step of CME has been demonstrated to involve phase separation. Whether the subsequent steps involve phase separation remain to be addressed. (c) ultrafast endocytosis rapidly generates large endosome from the plasma membrane. Dynamin accumulation via phase separation is implicated in the fast kinetics of the endocytosis. (d-e) following ultrafast endocytosis, clathrin-mediated budding is required to generate clathrin-coated vesicles. (f) uncoating of clathrin to produce empty SV. (g) neurotransmitter refilling. (h) SV re-entering the reserve pool. **B**. Domain organization and interaction among key proteins involved in CME nucleation and dynamin condensation. F-BAR, Fes/CIP4 homology BAR (Bin/amphiphysin/Rvs) domain; IDR, intrinsically disordered region; μHD, μ-homology domain; EH, Eps15-homology domain; DPF, tripeptide Asp-Pro-Phe (DPF) motif; UIM, Ubiquitin-interacting motif; SH3, SRC-Homology 3 Domain; DH, Dbl-homology domain; PH, Pleckstrin-homology domain; GED, GTPase effector domain; PRD, Proline-rich domain. Each double-head arrow indicates one pair of interaction.

containing membranes, both proteins showed homogenous coating on GUVs. Simultaneous attachment of FCHo1 and Eps15 on GUVs led to co-condensation of these two proteins,[205] and membrane attachment further lowered their threshold concentration for phase separation to nM range. These membrane-attached condensates are highly mobile and can rearrange their organizations.[205]

FCHo/Eps15 phase separation on GUVs nicely recapitulates the nucleation step of CME, whereby membrane binding is a prerequisite for the event to take place.

Dynamin phase separation for ultrafast endocytosis

About ten years ago, a new type of SV endocytosis was reported, with its speed about two orders of magnitude faster than typical CME. It takes as short as 50 milliseconds to retrieve membranes at the peri-active zone through the so-called ultrafast endocytosis.[209–210] Both actin polymerization and dynamin scission are indispensable for ultrafast endocytosis.[209] Dynamin plays critical roles in multiple pathways beyond CME.[195,211] Knocking out the brain-specific isoform(s) of dynamin severely impaired SV recycling, resulting in a great loss of SVs and accumulation of CCPs.[212–214] During vesicle scission, dynamin-mediated constriction occurs within hundreds of milliseconds.[215] However, it takes a few to tens of seconds to recruit them to membranes from cytoplasm,[216–217] suggesting there might be certain mechanism underpinning the placement of dynamin to its target site specifically for ultrafast endocytosis. It was counted that dozens of dynamins are assembled at the neck of a CCP, which may underlie the fast kinetics of membrane scission, though how this might happen was not known at the time.[217]

Double knockout of dynamins 1 and 3 in neurons increased the number of stalled endocytic pits, which can be fully rescued by the expression of dynamin 1.[218] Dynamin 1 accumulates at the endocytic sites, and behaves like liquid condensates.[218] Importantly, the liquid condensation behaviour and the restoration of ultrafast endocytosis depend on dephosphorylation of dynamin, implying a role for syndapin interaction in these processes as syndapin is the only known binding partner of dephosphorylated dynamin.[218–220] Similar to dynamin removal, syndapin knockout also impaired SV numbers via interfering with SV retrieval.[221] Syndapin contains an F-BAR domain, which binds to PIP2-containing membrane and induces negative membrane curvature. The F-BAR domain is autoinhibited by its C-terminal SH3 domain, which is released for membrane association when dynamin competes for binding to the same region.[222] In addition, syndapin itself can oligomerize via its BAR domain.[223] Together, syndapin provides a potential molecular explanation for the targeting and enrichment of dynamin on membrane surfaces.

Another intriguing finding was that dynamin 1xA, but not dynamin 1xB, formed puncta structure and restored the ultrafast endocytosis defects in dynamin 1, 3 double knockout mice.[218] Dynamin 1xA and dynamin 1xB are splicing variants of dynamin 1 and they share high sequence similarity including the binding sequence for syndapin. The only difference in sequence exists at the very C-terminal end: 1xA contains additional 20 residues covering multiple P-x-x-P motifs,[224] whereas 1xB contains a calcineurin binding motif (P-x-I-x-I-S).[225] Via its unique C-terminal tail, dynamin 1xA binds to endophilin with a higher affinity than 1xB, suggesting endophilin might be involved in the ultrafast endocytosis of SVs.[218] This is in line with an earlier report that endophilin and dynamin are mutually dependent for proper localization at endocytic sites.[226] Dynamin 1xA contains multiple PRMs for binding to multiple SH3-containing proteins like syndapin and endophilin. These SH3-containing proteins normally contain several SH3 domains connected in tandem or a dimerized BAR domain, which in return can enhance dynamin 1xA binding avidity.[227] Thus, one would predict that multivalent interactions may drive dynamin 1xA condensate formation at the endocytic sites to facilitate ultrafast endocytosis.

Perspectives

The wide presence of membraneless condensates in cells has expanded studies of biological macromolecules from the classical nano-/microscale level to the mesoscale level. Biomolecular condensates formed via phase separation possess unique features that are not observed in dilute and homogeneous systems, although we only know very little about the properties/behaviours of molecules in the condensed phase. From the very little that we do know now, many features specific to molecules in the condensed phase have already offered exciting insights for understanding numerous questions in broad areas of biology and medicine, providing new explanations to earlier experimental findings, reconciling many puzzling discoveries in the field, offering possible opportunities to target biological condensates for many diseases. In this review, we took the SV cycling in presynaptic bouton as the example to summarize and discuss the possible roles of phase separation in this specific cellular process. From what has been discovered to date, it is clear that phase separation is extensively involved in active zone formation, SV clustering and maintenance, SV exocytosis and endocytosis (Figure 2(B)). However, many detailed mechanisms governing how various condensates execute or regulate these processes are not known, and thus will be fertile research topics in the future. It is almost certain that many additional cellular processes and the malfunctioning of these processes are linked with phase separation and will be discovered in the coming years.

A distinct feature of biological condensates in SV recycling is that protein-based condensates intimately and extensively interact with membrane-based structures (e.g., vesicles and other organelles, plasma membranes) via different

modes. In fact, increasing evidence indicate that interactions between membraneless condensates and membranous organelles is a general feature within living cells. In addition to what has been discussed in this review, other examples include tight junctions,[228] apical and basal crescents,[229–230] and stereocilia tip-links[231–232] in polarized cells, postsynaptic density in neurons,[233] cellular focal adhesions,[234–235] T cell receptor signalling,[236] migrasome formation,[237] etc. Within a cell, stress granule condensates are connected to lysosomes for their co-transport along microtubules,[238] ER wraps around RNP granules and mediates their fission,[239] TIS granules intertwine with ER to control membrane protein translation,[240] ER resident protein STING phase separates to suppress innate immunity,[241] small vesicles co-assemble with proteins to transduce B cell antigen receptor signalling,[242] pre-autophagosomal structures interact with vacuolar membranes during apoptosis in yeast,[243] etc.

Second messengers and other small molecules such as Ca^{2+}, Mg^{2+}, cAMP, ATP, etc. are increasingly appreciated to regulate phase separations of broad ranges of condensates. These small molecules can regulate formation or dispersion of cellular condensates via modulating conformational changes of proteins and nucleic acids via direct binding, altering bindings to their targets, modifying chemical properties of macrobiomolecules via posttranslational modifications, etc. For example, in the postsynaptic density, Ca^{2+} influx weakens CaMKII's interaction with Shank3 but concomitantly enhances the interaction between the active CaMKII and NMDAR, resulting in CaMKII translocation from the Shank3 condensates to the NMDAR condensates.[244] In axon, Ca^{2+} triggers direct interaction between Annexin A11 and lysosome membrane components and mediates the attachment of RNA granules to lysosomes for trafficking along the microtubules.[238] In the G-protein coupled receptor-PKA signalling pathway, cAMP was shown to promote phase separation of PKA regulatory subunit – RIα.[245] Noticeably, this study used a fluorescently labelled cAMP analog, allowing direct visualization of this small molecule being enriched in the PKA-RIα condensates.[245] Small molecules has also been shown to directly modulate phase separation of various condensates. For example, high concentration ATP is a general negative regulator of phase separations of many different condensates.[246] Ca^{2+} and Mg^{2+} were recently shown to enhance α-synuclein phase separation ability and facilitate its liquid-to-solid transition,[247–248] though with unknown mechanisms. Another exciting direction of small molecule-mediated regulations of phase separation is to develop compounds with therapeutic potential by targeting biological condensates.[249–252]

We wish to emphasize that researchers should be extra cautious when working with phase separation in biology. Based on phase separation theory of polymers, the majority if not all protein and nucleic acid biopolymers can phase separate in aqueous solution if conditions (e.g., concentration, salt concentration and pH of buffers, additives, temperature, etc) are met. A vital point to keep in mind is whether a biomolecule or a molecular assembly in its physiological milieu and at cellular concentrations can undergo phase separation. A prevailing concept in the phase separation field up to date states or at least implies that intrinsically disordered sequences of proteins drive phase separation of biological condensates. We believe that this is a rather biased picture. There is no doubt that intrinsically disordered sequences play important roles in numerous proteins and in some cases play dominant roles. However, intrinsically disordered sequences are often required but not sufficient for functional biological condensate formation in cells. Specific interactions are vitally important for biological condensates to achieve specific cellular functions in right cellular locations at proper time points.[253–257] A more complete picture is likely that specific interactions mediated by modular domains and weak non-specific interactions mediated by intrinsically disordered sequences together govern most of the biological condensate formation and function in living cells. In line with this argument, although the human proteome contains a vast amount of intrinsically disordered sequences in ∼ 40% of proteins,[258] majority of these intrinsically disordered sequence-containing proteins contain one or multiple specific target recognition domains/motifs.

Finally, current theories and technologies developed for studying dilute and homogeneous solutions are often not adequate or even not applicable for studying biological condensates, which are better characterized as soft matters in condensed matter physics. There are urgent needs in developing new technologies and theories for the exciting filed of biological condensates.

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Abbreviations:
AP2, adaptor protein 2; BAR, Bin/amphiphysin/Rvs; CCP, clathrin-coated pit; CME, clathrin-mediated endocytosis; CT, C-terminal tail; Eps15, EGFR pathway substrate 15; EM, electron microscopy; FCHo, FCH domain only; FUS, fused in sarcoma; GUV, giant unilamellar vesicle; HSC70, heat shock cognate 70; ITSN, intersectin; Munc13-C, C1-C2B-MUN-C2C; PIP2, phosphatidylinositol-4,5-bipho sphate; PRM, proline-rich motif; RIM, Rab3-interacting molecule; RIM-BP, RIM-binding protein; RRP, release-ready pool or readily releasable pool; SLB, supported lipid bilayer; STED, stimulated emission depletion; STORM, stochastic optical reconstruction microscopy; SUV, small unilamellar vesicles; SV, synaptic vesicle; VGCC, Voltage-gated calcium channel; ZF, Zinc Finger

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