Trends in Neurosciences

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Review

Condensate biology of synaptic vesicle clusters

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Neuronal communication crucially relies on exocytosis of neurotransmitters from synaptic vesicles (SVs) which are clustered at synapses. To ensure reliable neurotransmitter release, synapses need to maintain an adequate pool of SVs at all times. Decades of research have established that SVs are clustered by synapsin 1, an abundant SV-associated phosphoprotein. The classical view postulates that SVs are crosslinked in a scaffold of protein–protein interactions between synapsins and their binding partners. Recent studies have shown that synapsins cluster SVs via liquid–liquid phase separation (LLPS), thus providing a new framework for the organization of the synapse. We discuss the evidence for phase separation of SVs, emphasizing emerging questions related to its regulation, specificity, and reversibility.

Highlights

Synapsins form fluid-like condensates enriched with synaptic vesicles (SVs).

The intrinsically disordered region of synapsin is necessary and sufficient for the formation of vesicle condensates.

The stoichiometry of synapsins and synucleins, two highly abundant synaptic proteins, is crucial for maintaining the architecture of SV condensates.

Synapsin-driven condensates are reversible upon phosphorylation.

Membrane properties and integral proteins of SVs drive the recruitment of vesicles into the phase and determine the motility of SVs between neighboring boutons.

SV clusters as an example of a liquid phase

LLPS is a process in which one or multiple components in the same physical state segregate from another component into distinct compartments, for example the demixing of oil in water. In the context of cell biology, it is a process where (bio)polymers separate from a homogeneous aqueous mixture by forming distinct structures that are often referred to as biomolecular condensates or membraneless organelles [1,2]. The process of phase separation is now known to facilitate many complex cellular functions, including DNA replication [3], transcription [4], signaling [5], and the formation of stress granules [6], to name a few, thus emerging as an essential mechanism for intracellular organization [7]. The crucial features of biomolecules that undergo phase separation are their high concentration and their ability to engage in multivalent, low-affinity interactions such as the interaction between SRC homology 3 (SH3) domains and proline-rich motifs [8,9]. Upon reaching the saturation concentration in a bulk solution, the so-called critical concentration, these biomolecules spontaneously assemble into a dense phase without any surrounding membrane or a scaffold [10]. Such a dense phase of biomolecules can spatially concentrate some molecules while excluding others [11]. Importantly, the mechanisms of phase separation are size-independent and are not restricted to nucleic acids and proteins but also include cohorts of organelles such as the stack of secretory vesicles [12], the Golgi apparatus [13,14], and the SVs [15–17]. Moreover, the biological activity of some biomolecules depends on their ability to form condensates. For example, in neurons, the formation and transport of RNA granules is tightly coupled to their ability to form biomolecular condensates [18,19]. The viscosity of the RNA granules determines the extent of their association with membrane-bound compartments such as lysosomes.

Many proteins implicated in condensate formation contain an intrinsically disordered region (IDR) [6,20]. IDRs are stretches of amino acids that do not fold into any stable secondary or tertiary structure. Interestingly, roughly a third of eukaryotic proteins contain IDRs of at least 50 amino acids in length [21]. Many proteins involved in SV recycling contain long IDRs [22]. Amino acid consensus sequences for binding to protein–protein interaction modules, such as proline-rich motifs, are often found within these regions. The presence of IDRs is essential for phase separation of these proteins [9,23], where particular amino acid patches modulate the recruitment of

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additional components into these condensates through both low- and high-affinity interactions [10].

Neurotransmission depends on the tightly regulated spatial and temporal release of neurotransmitters [24,25]. Neurotransmitters are packed into membrane-bound structures known as SVs. Nerve terminals contain hundreds of SVs which are tightly clustered [26,27]. SVs have the properties of a fluid phase in which vesicles are one component of the phase and the other is a protein of the interweaving matrix [16]. Electron microscopy (EM) data across a range of different synapse models [26–30] indicate that SV clusters occupy a distinct cytosolic territory that is segregated from surrounding membrane-bound organelles. However, for many years the precise molecular mechanisms that allow the sequestration of SVs into distinct cohorts remained unclear. It appears that SV clustering is independent of active zone proteins because deletion of the active zone components does not abolish upstream clustering of SVs [31,32].

Despite being held together in these clusters, vesicles are highly mobile such that they can be swiftly recruited to the neuronal plasma membrane to release their content upon activation of the neuron [33]. The intermixing of SVs has been observed in EM analyses of nerve terminals of the CNS and PNS following endocytic labeling with extracellular tracers and ligands of the luminal side of SV membranes [26,34,35]. Although a small fraction of SVs diffuse very rapidly and are actively recycled, the majority of SVs remain in the so-called 'reserve pool' [33]. However, the vesicles in the reserve pool are still mobile and their recruitment for release is very rapid upon both mild and strong stimulation [35–37]. This wide range in motility within synaptic boutons strongly suggests that SVs form a condensate rather than being crosslinked into a scaffold. Moreover, SVs in the dilute phase (i.e., outside the condensate) are able to rapidly exchange between neighboring terminals, and such an exchange of SVs along the axons has been reported [38,39].

Over the past several years many structures at the synapse have been described as examples of membraneless compartments (Figure 1), including the active zone [40–43], endocytic sites [44], and the postsynaptic density [45,46]. In this review we focus on SV condensates and emphasize new insights into their assembly and discuss emerging questions related to their specificity, reversibility, and function.

Synapsins, central regulators of SV condensates

Using lipid vesicles and purified proteins in combination with genetic analyses, it was recently shown that synapsin 1 is able to form biomolecular condensates [15,17]. Droplets of synapsin 1 have all the expected properties of a liquid phase: they fuse with each other and recover after photobleaching, suggesting swift exchange into and out of synapsin 1 droplets. In addition, synapsin 1 binding to scaffolding proteins such as growth factor receptor-bound protein 2 (Grb2) and intersectin further modulates this phase but is not necessary for its formation [15]. Importantly, synapsin 1 can capture small lipid vesicles into its phase, as confirmed by EM analyses: synapsin/liposome biocondensates contained clusters of small vesicles, whereas in the absence of synapsin 1, liposomes do not form such clusters. These data corroborate with analysis of living synapses upon both acute and chronic disruption of synapsins [15,17,47–49]. For example, injection of anti-synapsin antibodies into the giant reticulospinal synapse of the lamprey results in dispersion of SVs both at rest [17] and upon depolarization [48] (Figure 2A,B). Only a pool of vesicles more proximal to the active zones, most likely a pool crosslinked by other factors, remains. Similarly, chronic depletion of synapsins in mice – through synapsin gene deletions – results in the less vesicles accumulating at the synaptic bouton, and the SVs within the bouton are more dispersed than in wild-type synapses, both in cultured neurons and in brain sections (Figure 2C) [15,47,49,50].

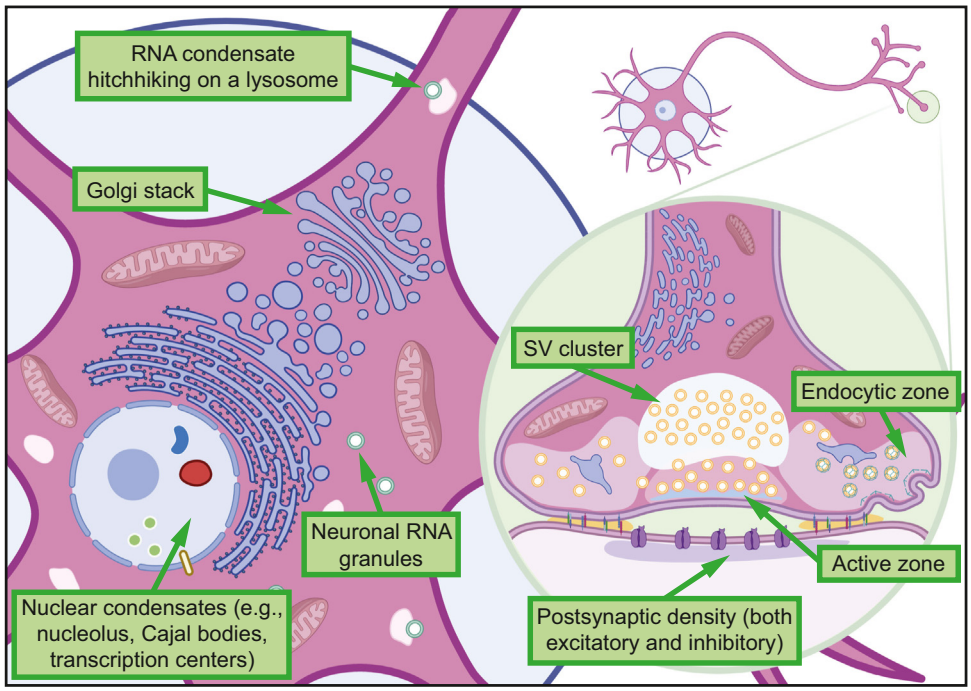

Figure 1. Biomolecular condensates as an organizing principle in neuronal and synaptic biology. Scheme of a neuron, and sites where biomolecular condensates may play a role in neuronal and synaptic function. Blue background circle: focus on the soma, where several structures in the nucleus (e.g., nucleolus, Cajal bodies) and cytoplasm (e.g., neuronal RNA granules, Golgi stack, secretory vesicles) are shown to assemble by liquid–liquid phase separation (LLPS). Green background circle: focus on the synapse, with an emphasis on several membraneless condensates both at the presynapse (e.g., SV condensates, active zone, endocytic sites) and postsynapse (e.g., excitatory PSD95-containing condensates, inhibitory gephyrin sheets). Abbreviation: SV, synaptic vesicle.

Quantitative analyses of synapses suggest that synapsin concentrations may exceed 120 μM [51]. Synapsins are encoded by three genes and represent a major synaptic family of phosphoproteins [52,53]. During the isolation of SVs from mammalian brains, synapsin 1 represents ~9% of total protein associated with SVs [54,55]. Synapsins do not affect the docking or fusion of SVs to the presynaptic membrane [47]. In addition, synapsins are not present on SV membranes during the endocytosis of SV components from the plasma membrane (e.g., endocytic intermediates such as clathrin-coated vesicles) [56].

A major property of the highly abundant SV-associated protein synapsin 1 is the presence of a large IDR. Notably, the IDR of synapsin 1 is responsible for its ability to phase-separate [15]. Synapsin 1, which is expressed at the highest levels of all three synapsins, appears in two nearly identical splice variants *a* and *b*, where isoform *a* encodes a slightly longer protein at the C-terminal region [57]. Specifically, amino acids 1–660 are precisely the same in both isoforms; isoform *a* contains amino acids 661–705 that differ from isoform *b* (amino acids 661–669) and is considered to be a predominant isoform. In murine hippocampal synapses, synapsin 1 contributes to the majority of expressed synapsins.

An additional feature of synapsins is their ability to homo- and hetero-oligomerize [58,59]. The oligomerization ability plays an important role in lowering the critical concentration of molecules implicated in condensate formation [60]. Another member of the synapsin protein family of

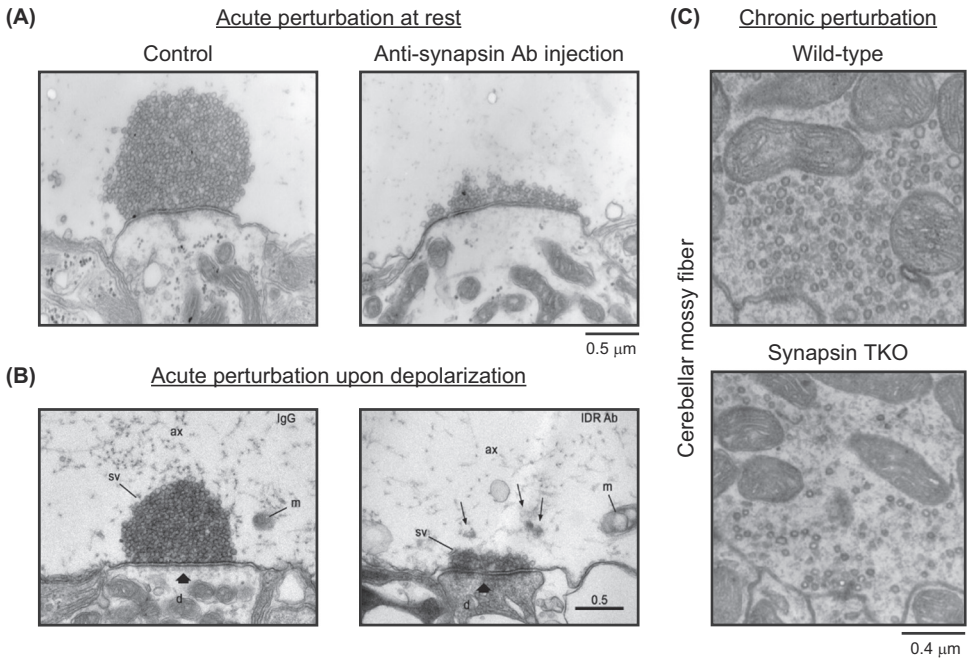

Figure 2. Acute and chronic depletion of synapsins results in the dispersion of synaptic vesicles (SVs) from the synaptic bouton. Electron microscopy (EM) images upon injection of anti-synapsin antibodies both at rest (A) and upon depolarization (B) in the giant axon of the lamprey show a full dispersion of SVs. Note that only SVs adjacent to the presynaptic plasma membrane remain, probably maintained by active zone proteins. (C) EM images of synapses from cerebellar mossy fibers obtained from adult wild-type (top) and synapsin triple-knockout (TKO, bottom) mice. Note that both the number and the packing of SVs at the synaptic bouton were reduced in synapsin TKO animals. Abbreviations: Ab, antibody; ax, axonal cytosol; d, dendrite; IDR, intrinsically disordered region; m, mitochondria. Figure modified, with permission, from [15,17,48].

three – synapsin 2 – has been shown to phase-separate, albeit at a slightly slower rate [15]. This tight interaction of synapsins may be important for the assembly of functional SV condensates. Indeed, synapsin 2a is the only synapsin isoform that can rescue the accelerated depression detected in neurons from synapsin triple-knockout (TKO) animals [49,61]. Interestingly, the IDR of synapsin 1 is nearly twofold longer than that of synapsins 2 and 3. Several features connect the IDRs of all three synapsins: (i) repetitive proline sequence elements spaced by lysines or arginines, (ii) serine/glycine/glutamine-rich regions, and (iii) their positive net charge. These features of the synapsin IDR and variations in its length between the different family members suggest possible modularity and raise the question of how exactly synapsin 1 IDR architecture contributes to its phase-separating properties and SV clustering.

Stoichiometry of synapsins and synucleins modulates SV condensation

Stoichiometry plays a central role in driving the dynamics, surface tension, and viscoelastic properties of biomolecular condensates [10]. Thus, proteins highly concentrated at the synapse are poised to affect SV condensation. Apart from synapsins, a highly abundant protein family in the nerve terminal is the family of synucleins (α-, β-, and γ-synucleins), which also lack a stable tertiary structure [62]. Moreover, during the past decades α-synuclein has been under the spotlight because of its involvement in neurodegenerative diseases collectively called synucleinopathies [63]. Independently of its role in neurodegeneration, α-synuclein is an intriguing synaptic protein for several reasons. First, it is highly abundant at the nerve terminal (~50 μM) [51]. α-Synuclein is structurally unfolded in solution, but forms an α-helical fold upon binding to negatively charged

membranes [64,65]. It has been shown to chaperone the formation of SNARE fusion complexes, thereby facilitating exocytosis [66,67]. Analyses of living synaptic boutons in both culture and minimal reconstituted systems show that α-synuclein is enriched at SV clusters [68,69]. Independently of SVs, pathologically high concentrations of α-synuclein can drive its aggregation through LLPS [70,71]. Finally, α-synuclein interacts with β- and γ-synucleins for binding to SVs [72], locally increasing the total concentration of synucleins at the SV surface.

Interestingly, genetic deletion of synucleins in animals results in the opposite phenotype than the deletion of synapsins (Figure 3A,B). *In situ* analyses of nerve terminals in mice that lack all three synucleins showed a more densely packed and highly ordered 3D arrangement of SVs than in the nerve terminals of wild-type mice (Figure 3C) [73]. Given that these synuclein TKO mice still expressed synapsin [74], which is essential and sufficient for SV cluster formation, the presence of SV clusters is not surprising. Synuclein TKO animals have smaller terminals [74] that could, at least in part, explain the tight packing; however, the highly ordered architecture of these clusters suggests a fundamental alteration of its material properties.

Intriguing functional connections between synapsin and α-synuclein have been reported. Overexpression of α-synuclein in wild-type murine synapses results in a decrease of SV release and recycling [75]. However, this phenotype is absent when α-synuclein is overexpressed in neurons derived from synapsin TKO animals [76]. This suggests that SV mobility and cluster density depend on a tight balance between the concentrations of synapsin and α-synuclein.

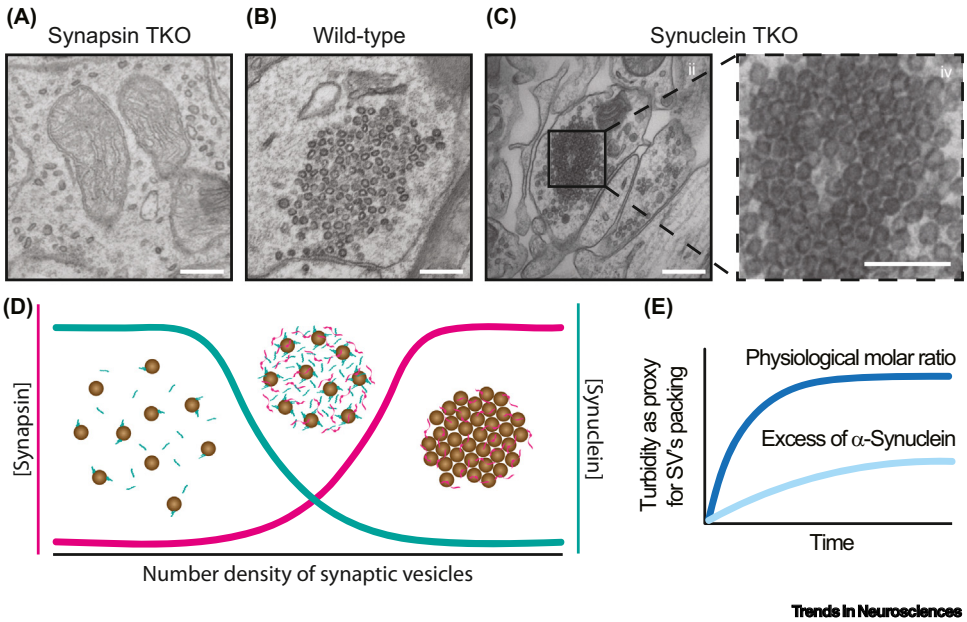

Figure 3. Synapsin/synuclein interaction affects the architecture of synaptic vesicle (SV) condensates. (A) Presynaptic terminal of synapsin triple-knockout (TKO) mouse containing dispersed SVs. (B) A nerve terminal of a wild-type mouse with the representative SV cluster. (C) SVs are tightly packed with a highly ordered structure in synuclein TKO synapses. (D) Scheme of SV condensation in the presence of different concentrations of α-synuclein and synapsin. (E) Excess α-synuclein reduces the rate of synapsin condensate formation. Condensate formation by purified recombinant synapsin 1 and α-synuclein in different molar ratios (curves in tones of blue). Condensate formation was measured by a change in turbidity. Scale bars: A, 0.3 μm; B, 0.2 μm; C, 0.4 μm; C inset, 0.2 μm. Figure modified, with permission, from [15,16,73,77].

The large synapsin 1 IDR with numerous proline-rich motifs enables fast condensation starting at lower nucleation threshold concentrations [15]. Given the basic pI, it is tempting to speculate that synapsin 1 IDR acts as a molecular filter that can selectively retain SVs through multivalent, low-affinity interactions with acidic clients – the integral and peripheral SV proteins. Indeed, recent results suggest that α-synuclein, which contains a negatively charged tail, accumulates in synapsin condensates to form a liquid phase [77]. The appearance of these droplets was dependent on the amount of transfected plasmid, suggesting that there is a threshold concentration of synapsin 1 above which demixing starts, resulting in the formation of liquid condensates that actively sequester α-synuclein.

Interestingly, in the reconstitution system, the molar ratio of synapsin 1 and α-synuclein, affects the kinetics of SV phase separation: excess of α-synuclein decreases the overall turbidity of synapsin/SV condensates (Figure 3D,E) [77]. Considering turbidity as a proxy for molecular packing, these data indicate that high concentrations of α-synuclein attenuate the phase separation of SVs. This corroborates with data from transgenic murine models [78]. In fact, a recent EM analysis of acute depletion of α-synuclein in the large synaptic boutons of lamprey using injection of anti-synuclein antibodies indicated piecemeal disruption of the SV phase in a dose-dependent manner [79]. Of note, this scenario contrasts sharply with the full dispersion of SVs in the same experimental model upon injection of anti-synapsin antibodies (Figure 2) [17]. Together, these *in vivo* results corroborate with the reconstitution data, and strongly suggest that α-synuclein plays an important role in higher-order mesoscale assembly of SVs in a concentration-dependent manner. Although the current experimental data focus on synucleins and synapsins, it is important to note that the role of stoichiometry (i.e., molar ratio) of proteins in SV condensates may not be limited to these two proteins. The dose-dependent effects of other synaptic proteins enriched at the SV condensates remain to be determined.

Specificity of SV condensates: the roles of proteins?

SV condensates are mostly enriched with SVs and depleted of other organelles [27,30]. Membrane infoldings and endosomal structures are occasionally encountered in SVs, but mitochondria and clathrin-coated vesicles are absent from these condensates [80]. Thus, a major question emerges concerning how the specificity of SV/synapsin/α-synuclein condensates is achieved in recruiting a particular subset of organelles and proteins.

The cytosolic tails of integral SV proteins are poised to affect LLPS by modulating the overall valency and affinity of SVs for the phase (Figures 4 and 5). One such example is vesicle-associated membrane protein 2 (VAMP2), an integral protein of SVs that interacts with α-synuclein. At high protein-to-lipid molar ratios, α-synuclein and VAMP2 may increase the clustering of liposomes [81], but alone are not sufficient to induce mesoscale condensation of native SVs [77]. Although synapsin TKO animals still contain unchanged levels of α-synuclein and VAMP2, they lack mesoscale SV clusters [15]. Another representative example is a cytosolic tail of vesicular glutamate transporter 1 (VGLUT1; aa 491–560) – a glutamate transporter in excitatory synapses – that also has a net negative charge (pI 4.2). VGLUT1 is well known to regulate SV density at synaptic boutons [82–84], a phenotype not seen for VGLUT2 [85]. This differential effect appears to be due to the presence of poly-proline tail that interacts with the SH3 domain-containing protein endophilin A1 [86]. In fact, a recent study showed that disrupting the multivalent, low-affinity interactions of the cytosolic tail of VGLUT1 enhanced the exchange of vesicles between neighboring synapses [87]. These observations are linked to functional changes in release probability, short-term synaptic plasticity, and spontaneous miniature release frequency [87,88].

Finally, a third example is the cytosolic tail of the SV-resident protein synaptophysin (aa 219–307, rat sequence) that has a low-complexity and acidic charge (pI 3.9). At the neuromuscular junction,

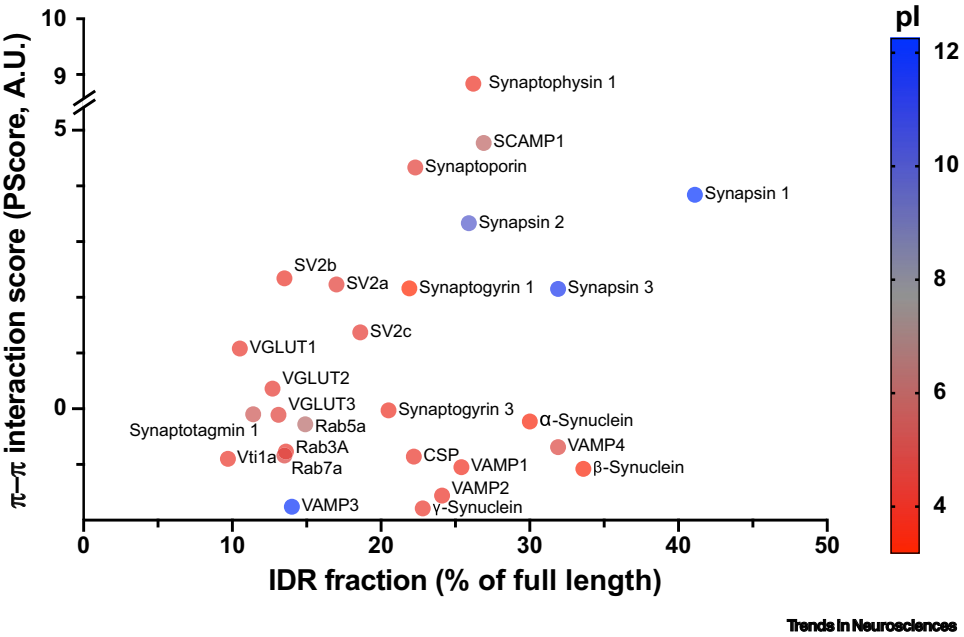

Figure 4. Summary of chemical characteristics for peripheral and integral synaptic vesicle (SV) proteins. For each protein three parameters were assigned: the fraction of the intrinsically disordered region (IDR) with respect to total protein length [106], the potential to form π–π interactions [108], and the isoelectric point of their IDRs. Note the sharp distribution of charges and that only a few proteins are able to form π–π interactions. Abbreviation: A.U., arbitrary units.

the bulk of synapsin 1 remains associated with synaptophysin-positive membranes during the exo/endocytic cycle [54]. Tetraspan membrane proteins of SVs include three major families: synaptophysins (1 and 2), synaptogyrins (2 and 4), and secretory-carrier membrane proteins (1–3). Of these, synaptophysin 1 is the most abundant tetraspan SV protein, representing 10% of total SV protein content [89], and contains a C-terminal region of low structural complexity that is exposed to the cytosol [90,91]. Only four amino acids (Gly, Pro, Gln, Tyr) compose >50% of the sequence of C-terminal region of synaptophysin [92]. Indeed, synaptophysin contains an acidic tail that is sufficient and necessary for its reversible recruitment into the synapsin phase [93,94]. Ectopic expression of synaptophysin alone results in the formation of small SV-like vesicles in non-neuronal cells [95–97], but coexpression of synaptophysin and synapsin 1 results in the formation of vesicular condensates [93].

The physiological effects of knocking out the tetraspan membrane proteins of SVs are relatively modest [98–100]. Only the deletion of four major members – synaptophysin 1, synaptophysin 2/synaptoporin, synaptogyrin 1, and synaptogyrin 3 – results in an increased release frequency under mild stimulation [101]. This physiological phenotype that requires deletion of all major members is puzzling and suggests that redundant mechanisms have evolved to balance the organization of SVs at the nerve terminal. EM images show that the deletion of synaptophysin and synaptogyrin in flies results in SVs of varying diameter [102]. This indicates an intricate relationship between the presence of tetraspan membrane proteins and bilayer properties, which in the case of synaptophysin affects the recruitment of vesicles into condensates.

SV cohorts are, in fact, a heterogeneous population of organelles with distinct molecular signatures (see [103] for details). For example, specific SV proteins that favor either synchronous or asynchronous release are poised to have a differential effect on SV dynamics within condensates

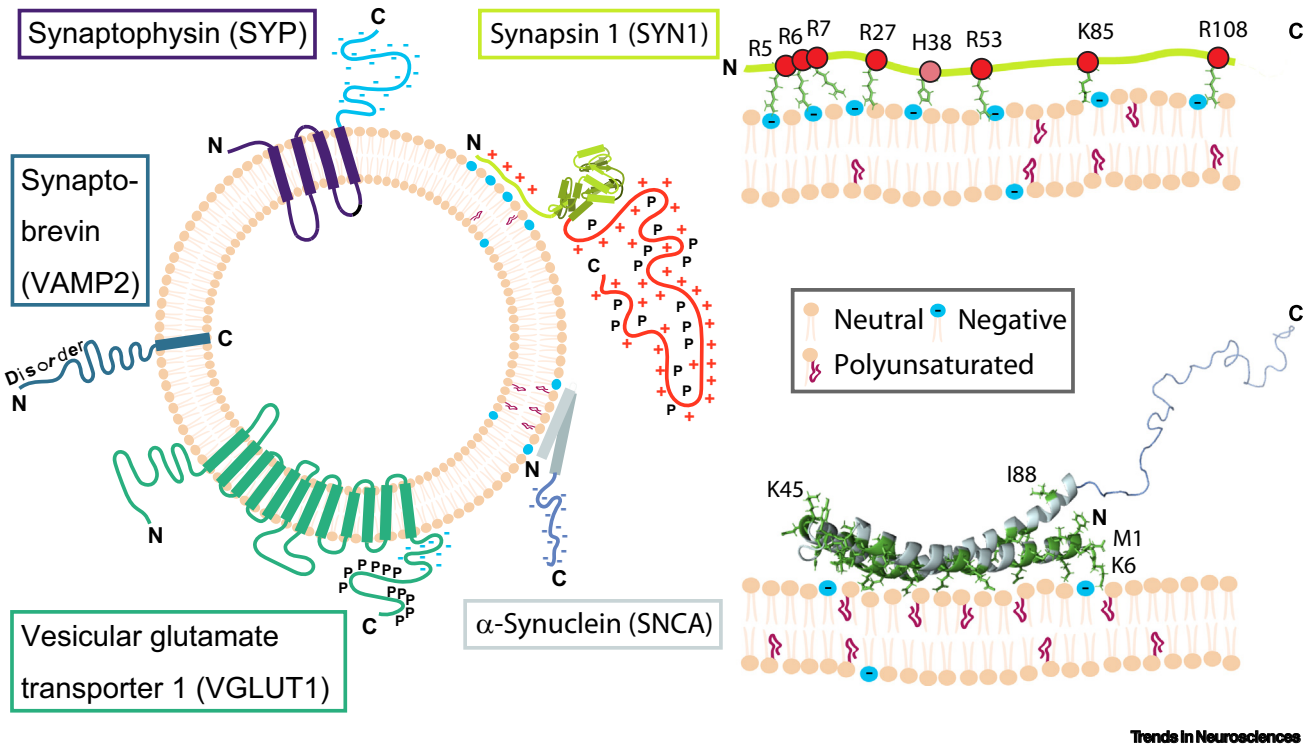

Figure 5. The specificity of synaptic vesicle (SV) condensates is determined by the surface properties of integral proteins and lipids. Scheme of interactions between SV, synapsin, and α-synuclein. (Left) Integral proteins of SVs that are experimentally shown to affect SV condensates. (Right) The N-terminal region of synapsin 1 (top) and the aliphatic helix of α-synuclein (bottom; PDB:1XQ8) can interact with lipids with different charges and level of saturation.

[104,105]. Based on the presence of IDRs [106], charge patterns [107], and the probability of amino acids to engage in π–π interactions [108], Figure 4 summarizes the chemical features of SV proteins that could modulate SV condensation. Systematically addressing the effects of these proteins on synaptic physiology requires careful replacement of disordered patches with ectopic regions of proteins that are well documented to form condensates [109].

Although VAMP2, synaptophysin, and VGLUT1 alone are insufficient to trigger LLPS of SVs, they might play a central role in regulating the specificity and partitioning of SVs into condensates. Therefore, systematic delineation will be necessary to determine how distinct membrane features (i.e., surface charge, lipid packing, and curvature) and the presence of specific proteins determine specificity of SV clusters at synapses (see Outstanding questions).

Specificity of SV condensates: the roles of lipids?

Although synapsin undergoes LLPS by itself, the incorporation of SVs augments this process [77]. The acidity of SVs seems to play an important role in synapsin/lipid vesicle condensation (Figure 5). For example, the incorporation of phosphatidylserine suffices to include artificial liposomes in synapsin condensates [15] whereas neutral liposomes have no effect on enhancing condensation [15,41]. Synapsin 1 interacts readily with negatively charged phospholipids [110]. Similarly, the affinity of α-synuclein for negatively charged phospholipids [111–113] might be of crucial importance to locally enrich α-synuclein into synapsin/SV condensates. Furthermore, SVs contain large amount of polyunsaturated fatty acids (PUFAs), and this property was shown to play an essential role in facilitating vesicle remodeling during the exo/endocytic cycle [114].

How the local enrichment of PUFAs, and thus membrane packing, affect the binding of proteins to the bilayer of SVs remains to be investigated.

Interestingly, with a diameter of ~45 nm [89], SVs are among the smallest organelles, which implies that they have a pronounced curvature. Indeed, synapsins contain an amphipathic lipid packing sensor (ALPS) motif within their membrane-binding region, which is evolutionary conserved and contains many polar hydrophilic and hydrophobic residues [115]. ALPS can fold into amphipathic α-helices upon membrane contact that recognize local membrane deformations, such as in the case of highly curved SV membranes [116]. Although disordered in solution, α-synuclein is able to bind to the highly curved SVs, forming a broken helix at its N-terminal end, or to flat membranes where it forms an extended helix [64,65], suggesting that curvature might be important for α-synuclein recruitment to the surface of SVs.

Although the individual interactions of synapsins and synucleins with the lipid bilayer are well characterized, it remains unknown how binding of α-synuclein to the lipid bilayer is affected by the presence of synapsins – major proteins that are highly enriched on SVs. Interestingly, α-synuclein forms oligomers on the surface of SVs [67] and α-synuclein oligomerization accelerates the fusion reaction during neurotransmitter release.

Much of the evidence discussed in the previous section is circumstantial, and it is important to note that none of these features are exclusive to SVs. SVs contain large amounts (~20 mol%) of negatively charged phospholipids, particularly phosphatidyl serine [89]. Apart from SVs, however, there are numerous other organelles (e.g., lysosomes, the inner leaflet of the plasma membrane) that are negatively charged [117]. Hence, the specificity cannot be explained solely by the charge of the membrane. Similarly, curvature and local packing defects in the membranes appear across different organelles. Nevertheless, the combination of these properties might at least in part explain the specificity of SV/synapsin/α-synuclein condensates in recruiting only a subset of membrane-bound organelles, urging further studies of the molecular determinants of SV phase assembly (see Outstanding questions).

Reversibility of SV condensates: the effects of phosphorylation?

A key feature of liquid condensates is their potential reversibility under physiological conditions, particularly through post-translational modifications. During depolarization, numerous kinases and phosphatases jointly regulate the cascade of de/phosphorylation reactions at >250 protein sites [118], most of which are located in proteins responsible for SV release. Numerous kinases and phosphatases are involved, including calcium-dependent activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA), protein kinase C (PKC), protein phosphatases 2A and 2B (PP2A and PP2B); at the same time there is downregulation of protein phosphatase 1 (PP1) and proline-directed kinases such as cyclin-dependent kinase 5 (Cdk5) and glycogen synthase kinase 3 (GSK-3).

Phosphorylation events at the synapse have been shown to actively regulate SV clustering. Inhibition of kinases and phosphatases that have been linked to vesicle clustering via synapsin increases the inter-bouton mobility of SVs [50,119]. Synapsins are thought to be the prime targets of phosphorylation at the presynapse [120]. Synapsin contains multiple phosphorylation sites. Interestingly, although some phosphorylation sites are conserved between the three synapsins and also during evolution, others appear to be exclusive to mammalian synapsin 1. For example, the PKA site (Ser9 in human synapsins 1 and 2) is well conserved among both vertebrates and invertebrates, whereas only mammalian synapsin 1 has specific phosphorylation sites for CaMKII in its C terminus (Ser568 and Ser605 in the human sequence) [121].

In mammalian synapses, the classical view of synapsin regulation during neuronal activity considers two main and opposite phosphorylation patterns (Figure 6). During rest, sites 4–6 (Ser62, Ser67, and Ser551 in the human sequence) are constitutively phosphorylated by mitogen-activated protein kinase (MAPK), and this process is under the control of brain-derived neurotrophic factor [122]. Upon neuronal stimulation, the influx of calcium ions activates calcineurin/PP2B phosphatase that removes the phosphate groups from sites 4–6 [123]. At the same time PKA and CaMKII kinases phosphorylate sites 1 (Ser9) and 2–3 (Ser568 and Ser605), respectively, leading to reduced SV binding [124].

Subsequently, once the stimulation is over, phosphorylation of site 8 (Tyr301 in human sequence) in domain C by proto-oncogene tyrosine-protein kinase Src positively promotes synapsin 1 dimerization and binding to SV and actin, thus promoting reclustering of recycled SVs [125,126]. Moreover, Cdk5 phosphorylates sites 6 and 7 (Ser551 and Ser553 in human sequence), further enhancing SV clustering [127]. Beyond synapsins, it has been suggested that the balance of Cdk5 and calcineurin activity controls the portioning between the reserve and recycling pool of SVs [128]. Recently, ataxia telangiectasia mutated (ATM) kinase has been shown to localize at SVs and phosphorylates the extreme C terminus of synapsin 1 (Ser683 in the human sequence) [129], which is well conserved among both vertebrates and invertebrates. Although the functional role of ATM kinase has not yet been characterized, to our knowledge, genetic depletion of ATM is associated with deficits in spontaneous vesicular release and with neurodegeneration [129].

In fact, the synapsin and lipid vesicle phase rapidly disassembles upon phosphorylation by CaMKII [15], mimicking the dispersion of synapsin 1 that occurs at presynaptic boutons upon simulation [130,131]. Post-translational modifications such as phosphorylation can thereby regulate SV condensates, thus resembling the regulation of other biomolecular condensates [132] by changing the thermodynamic properties of the proteins involved in phase separation [133]. However, synapsins are targets for numerous additional kinases and phosphatases. These dynamic phosphorylation events of synapsin are important for balancing excitatory and inhibitory synaptic transmission and short-term plasticity [134,135]. Moreover, neuromodulators can also control the overall number of SVs at the synapse through synapsin phosphorylation [136].

This exemplary and incomplete catalogue of individual enzymes and their target sites on synapsin 1 already raises questions of how they collaborate together, whether there is a competition between

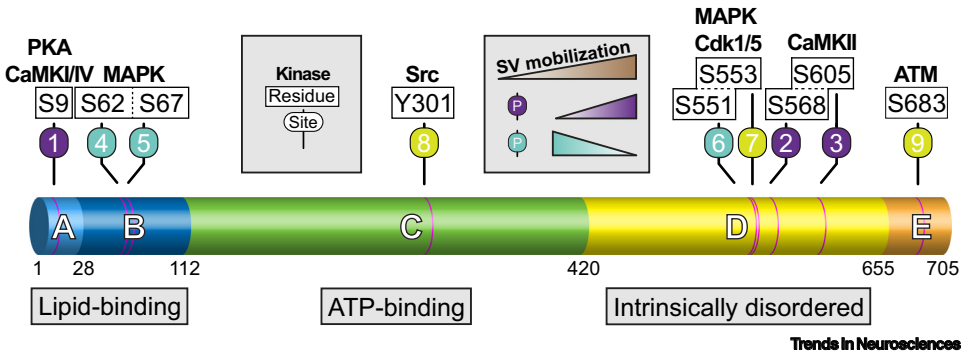

Figure 6. Phosphorylation of synapsin 1 is dynamically regulated during the synaptic vesicle (SV) cycle. Scheme of synapsin 1 domain organization (A–E). Sites (1–9) for different kinases are highlighted in circles. Purple circles represent sites that are phosphorylated upon depolarization; green and turquoise represent sites that are phosphorylated at rest. Residue numbers are according to the human sequence. Abbreviations: ATM, ataxia telangiectasia mutated; MAPK, mitogen-activated protein kinase; PKA, protein kinase A.

different sites, and which of these target sites are stoichiometrically phosphorylated (see Outstanding questions). Understanding the interplay of these enzymes will be essential for understanding SV phase dynamics during presynaptic long-term potentiation and their dysfunction in pathology.

Moreover, phosphorylation of α-synuclein (e.g., Tyr125, Ser129, Tyr133, Tyr136) is relevant for synaptic functioning and membrane binding, and has been linked to the formation of insoluble fibrils, a hallmark of Parkinson's disease [63,137]. It is probable that phosphorylation and/or dephosphorylation of different domains of synapsin 1 and/or α-synuclein regulate both their condensation and binding to SVs, but how this takes place is unclear. Although a multimolecular protein scaffold would require reassembly to release and sequester SVs, an SV liquid phase would allow dynamic sequestration both at rest and upon stimulation. Thus, an integrated view on how these distinct signaling events converge will be essential for understanding the dynamic regulatory network of protein–protein and protein–vesicle interactions that generate a liquid phase.

Concluding remarks

The organization of SVs into liquid condensates functionally impacts on synapses in several ways. First, SV clusters can actively incorporate proteins of the exo/endocytic cycle [138], acting as a buffer for synaptic proteins [139]. An SV condensate would allow many synaptic proteins to be transiently enriched in this phase as client proteins through specific protein–protein interactions. Indeed, many of the proteins involved in the SV cycle (e.g., amphiphysin, endophilin, intersectin, to name a few) contain SH3 domains that can interact with the proline-rich regions present within the IDR of synapsin. For low-affinity interactors in particular, the presence of a dense phase (e.g., an SV condensate) could increase the dwell time of molecules entering the phase, thereby allowing for their local enrichment [140]. Second, a liquid phase of synapsin and SVs at the presynapse allows the accumulation of incoming SVs from the cell body against the concentration gradient, which is particularly important given that the volume of an average button is 1000-fold smaller than the volume of an average axon [51]. Finally, a liquid condensate of SVs allows the release of a large range of vesicles depending on stimulation strength and duration, while the remaining SVs are maintained as a cluster [103].

Together, the concept of LLPS of SVs provides a new framework to look at the organization of the synapse, and it lays out testable experimental hypotheses regarding the specificity, reversibility, and dynamics of the SV phase (see Outstanding questions). Moreover, the SV phase interacts with the neighboring active zone, sites of endocytosis, and numerous membrane-bound organelles. How these interactions are regulated to ensure the high spatial and temporal fidelity of the SV cycle remains to be determined.

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Declaration of interests

The authors declare no competing interests.

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Outstanding questions

How do different synapsin phosphorylation events modulate its ability to phase-separate and bind to SVs? In mammalian synapses, synapsins are targets for numerous kinases and phosphatases. It remains unclear how these different phosphorylation sites affect the LLPS properties of synapsin, and whether some phosphorylation sites have a dominant effect on SV condensation in neurons.

What are the molecular determinants that ensure the specificity of synapsin condensates for SVs? SV condensates are mostly composed of SVs and are depleted of other organelles. Large organelles such as mitochondria and the endoplasmic reticulum (ER) are completely devoid of these condensates. SVs are small, highly curved structures that contain high concentrations of PUFAs, implying that there are local packing defects and high bilayer disorder. Similarly, several integral proteins are enriched in the membrane of SVs. How SV condensates manage to only recruit a subset of organelles and soluble proteins remains unclear.

Which mechanisms regulate the interface of SV condensates with the surrounding regions? It is emerging that the active zone and endocytic sites at the presynapse also represent distinct liquid phases. Moreover, SV condensates are often surrounded by intracellular membranes of the ER and mitochondria as well as the plasma membrane. The condensates can generate capillary forces that can remodel surrounding membranes. How the SV condensate affects the neighboring condensates and the nanoscale organization and dynamics of lipids in the surrounding membranes remains elusive.

How do condensates of synapsins/SVs affect actin dynamics? Synapsins, together with SVs, lower the critical concentration of actin required for assembly of filaments. However, the functional role of actin in SV clustering at rest and upon activity is still unclear. It remains to be clarified whether there is competition between the regions of synapsin that drive SV condensation and the regions that facilitate actin polymerization.

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