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TOPICAL REVIEW

A role for synapsin tetramerization in synaptic vesicle clustering

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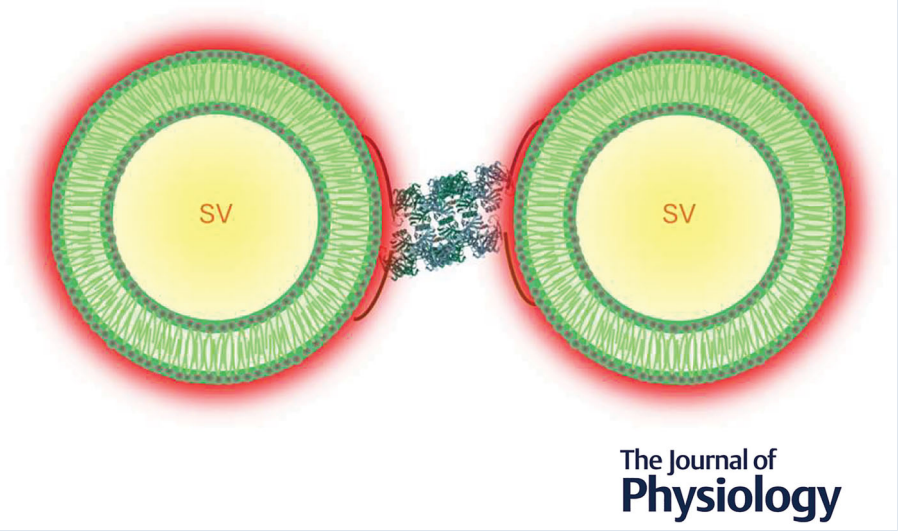

Abstract Although synapsins have long been proposed to be key regulators of synaptic vesicle (SV) clustering, their mechanism of action has remained mysterious and somewhat controversial. Here, we review synapsins and their associations with each other and with SVs. We highlight the recent hypothesis that synapsin tetramerization is a mechanism for SV clustering. This hypothesis, which aligns with numerous experimental results, suggests that the larger size of synapsin tetramers, in comparison to dimers, allows tetramers to form optimal bridges between SVs that overcome the repulsive force associated with the negatively charged membrane of SVs and allow synapsins to form a reserve pool of SVs within presynaptic terminals.

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Abstract figure legend Model depicting how synapsin oligomers could serve both as a long-range linker that interconnects synaptic vesicles to form a reserve pool of vesicles and as a spacer that circumvents short-range electrostatic repulsion between the vesicles.

Introduction

Neurotransmitters are stored within synaptic vesicles (SVs) and are released at presynaptic terminals via exocytotic fusion of SVs with the presynaptic plasma membrane. SVs are organized into distinct pools (Rizzoli & Betz, 2005). These include a readily-releasable pool (RRP) that represents SVs available for immediate exocytosis in response to presynaptic activity, in addition to a substantial reserve pool (RP) that supplies SVs to the RRP during prolonged activity (Fernandez-Busnadiego et al., 2010; Hilfiker et al., 1999; Siksou et al., 2007; Zuber & Lučić, 2019). This well-defined compartmentalization of SVs enables neurotransmitter release to be sustained robustly during synaptic activity by mobilization of SVs from the RP to the RRP and by subsequent exocytosis of neurotransmitters from these newly-delivered SVs.

Many of the proteins involved in SV trafficking have been identified and characterized (Augustine et al., 1999; Milovanovic & Jahn, 2015; Rothman et al., 2023; Südhof, 2013; Takamori et al., 2006). Among these proteins, synapsins have been identified as key regulators of SV dynamics within presynaptic terminals (Cesca et al., 2010; Hilfiker et al., 1999; Longhena et al., 2021; Song & Augustine, 2015; Zhang & Augustine, 2021). Synapsins were discovered in Paul Greengard's laboratory in the 1970s and hold a revered position as the first presynaptic protein to be identified and the first presynaptic protein to be implicated in SV trafficking (Greengard, 1978; Greengard et al., 1993). As a result, a wide range of experimental efforts have been invested in understanding synapsins and their functions (Atias et al., 2019; Gitler, Takagishi et al., 2004; Longfield et al., 2024; Milovanovic et al., 2018; Monaldi et al., 2010; Orlando et al., 2014; Song & Augustine, 2023).

From a molecular perspective, synapsins are fascinating because they are the most abundant protein kinase substrates in the brain, with phosphorylation by multiple kinases inducing dramatic and reversible changes in the biochemical properties of synapsins (Hosaka et al., 1999; Huttner et al., 1981; Kohansal-Nodehi et al., 2016; Song & Augustine, 2015; Takamori et al., 2006). Recent studies have shown that synapsins have intrinsically disordered regions that allow them to undergo liquid–liquid phase separation (LLPS) that might create membrane-less compartments within presynaptic terminals (Milovanovic et al., 2018). Also important is the dual ability of synapsins to bind to SVs reversibly (Hosaka et al., 1999; Huttner et al., 1981) and to oligomerize with each other (Brautigam et al., 2004; Hosaka & Sudhof, 1999). As will be emphasized in this review, together these properties are fundamental for synapsins to cluster SVs within the RP and to participate in mobilization of SVs from the RP to the RRP (Song & Augustine, 2023; Zhang & Augustine, 2021).

Structures of SV clusters and synapsins

The distinct pools of SVs can be identified by examining the kinetics of exocytosis (Rizzoli & Betz, 2005). The RRP is very small and represents the few SVs that are primed for rapid, synchronous release of neurotransmitters via exocytosis; the RP is the largest pool and represents SVs that undergo exocytosis only during periods of high synaptic activity; and the recycling pool is an intermediate-sized pool of SVs that repeatedly undergo exocytosis and are then locally reconstituted to sustain neurotransmitter release during low synaptic activity. Electron microscopy (EM) reveals that the RRP has a clear morphological correlate; it consists of SVs that are docked at (in close apposition to) the active zone (pink SVs in Fig. 1*A*; Imig et al., 2014; Schikorski & Stevens, 2001; but see Kaeser & Regehr, 2017). Although the location of the RP is less clear-cut, treatments that impair the RP selectively reduce the number of SVs distal to the active zone (Gitler, Takagishi et al., 2004; Pieribone et al., 1995). Thus, it is likely that most of the distal SVs that are not docked at the active zone constitute the RP (blue SVs in Fig. 1*A*). Their location and availability for exocytosis are the main criteria that distinguish SVs in the RP and RRP; no other structural features are known to distinguish these vesicle pools (Hilfiker & Augustine, 1999; Rizzoli, 2014; Song & Augustine, 2015). Synapsins have primarily been implicated in clustering of SVs in the RP (Gitler, Takagishi et al., 2004, 2008; Hilfiker et al., 1998; Song & Augustine, 2023; Vasileva et al., 2012), although there are suggestions that they might also influence exocytotic release of neurotransmitters from the RRP (Hilfiker et al., 1998; Humeau et al., 2001; Medrihan et al., 2013; Song & Augustine, 2016).

When viewed through the lens of EM tomography, the cluster of SVs within the presynaptic RP appears to be ensnared in a dense filamentous web (Fig. 1*A*; Cole et al., 2016). Three-dimensional reconstruction reveals four types of filaments, including two distinct types of filaments within the SV cluster (Fig. 1*B*). The larger type of filament within the SV cluster (gold in Fig. 1*B*; 74 nm in length and 5 nm in diameter) extends outward from above the active zone and into the vesicle cluster, while smaller filaments (purple in Fig. 1*B*; 30 nm in length and 8 nm in diameter) appear to form bridges between neighbouring SVs (Cole et al., 2016). These filaments, in particular the small filaments, presumably are responsible for clustering SVs within the RP. Very similar small bridging structures are visible between SVs in the presynaptic terminals of rat cerebellar mossy fibres (Fig. 2*A*; Hirokawa et al., 1989), rat hippocampal neurons (Fig. 2*B–D*), mouse hippocampal neurons (Fig. 2*E*; Wesseling et al., 2019), frog neuromuscular junctions (Heuser & Reese 1973), *Torpedo* electric organs (Hirokawa et al., 1989) and mouse cerebellar parallel fibres (Landis et al., 1988). The remarkable similarity of these bridge structures across a diverse array of synapses suggests that the bridges have a fundamental role in synaptic transmission.

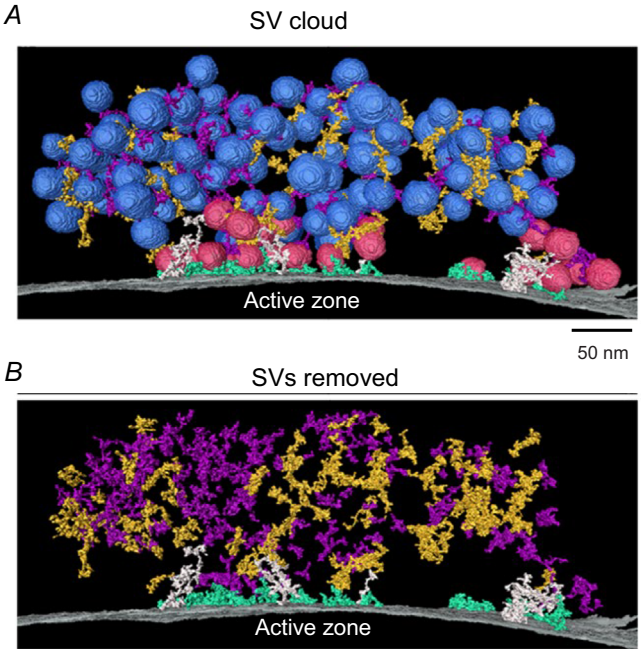

Figure 1. Three-dimensional rendering of electron microscopic tomography images of an active zone of rat hippocampal neurons
A, full complement of presynaptic structures; blue indicates synaptic vesicles (SVs) within a cluster; red indicates SVs docked at the active zone. *B*, same field as in *A*, with SVs removed digitally to allow better visualization of presynaptic filaments. Shown in purple are small filaments within the SV cluster that form bridges between SVs. Gold indicates large filaments within the SV cluster; these connect to SVs via side branches. White filaments extend from the active zone membrane and make contact with SVs in close proximity to the active zone, and teal filaments extend from the active zone membrane and interact with docked SVs. From Cole et al. (2016), reproduced with permission of the authors.

Although the molecular identity of these filaments is not entirely clear, EM has provided evidence that synapsins form intervesicular bridges. Individual synapsin 1a molecules resemble tadpoles, with a 15-nm-diameter globular head, from which a thin tail extends (Fig. 2*F*; Hirokawa et al., 1989; Landis et al., 1988). The apparent length of these synapsin molecules is between 35 and 50 nm, although these dimensions might be influenced by the specimen preparation procedures used. Mixing SVs with synapsin 1a yields structures that bridge neighbouring SVs, similar to those shown in Fig. 2*A–E*, and resemble the isolated synapsin molecules shown in Fig. 2*F*. These bridging structures range from 30 to 60 nm in length and appear to be two or more synapsin molecules that are attached by their globular head domains (Hirokawa et al., 1989). Furthermore, an EM tomographic comparison of synapsin wild-type (WT) and synapsin 1/2 double knockout (DKO) mice (Fig. 2*G*) shows that the synapsin-deficient presynaptic terminals of DKO mice have fewer bridges between SVs (Fig. 2*H*; Wesseling et al., 2019). Further analysis indicates that the bridges are strong enough to resist forces induced by chemical fixation, suggesting that these bridges both separate SVs and restrict their mobility (Wesseling et al., 2019). This is consistent with single-molecule tracking of SVs in hippocampal neurons, which indicates that the mobility of SVs within the RP is restricted by synapsins (Longfield et al., 2024). In conclusion, both EM and live-cell imaging studies indicate that synapsins can form strong bridges between SVs in physiological conditions.

How synapsins bind to SVs

Given the likely role of synapsins in forming bridges between SVs, it is important to understand how synapsins bind to SVs. Binding of synapsins to SVs is also important for synapsin localization to presynaptic terminals (Gitler, Xu et al., 2004). Synapsins can bind both to acidic phospholipids in the SV membrane and to proteins on the SV surface (Benfenati et al., 1989, 1993; Thiel et al., 1990). Synapsins are composed of a number of distinct domains (Fig. 3, inset; Hilfiker et al., 1999; Song & Augustine, 2015; Sudhof et al., 1989); the globular head is primarily composed of the large C domain, while the other domains form the tail structure. Several of these domains participate in the association of synapsins with SVs. Domain A, either alone or in combination with the B and C domains, is reported to bind to SV-like liposomes consisting of acidic phospholipids (Hosaka et al., 1999). This suggests that the A domain partly mediates binding of synapsins to SVs. The B

domains possess an amphipathic lipid-packing sensor motif that enables binding of synapsins to the tightly curved membranes of SVs (Krabben et al., 2011). Hydrophobic peptide fragments from the C domain bind to SVs and insert into the SV membrane (Cheetham et al., 2001). However, full-length C domain does not bind to liposomes (Hosaka et al., 1999), making it unlikely that C domain directly binds to SVs. The E domain further stabilizes the interaction of synapsins with the SV membrane (Monaldi et al., 2010).

As mentioned above, binding of synapsins to SVs is reversible and appears to be regulated tightly by protein kinases. Phosphorylation of the A domain by CaMKI (Ca^{2+}/calmodulin-dependent protein kinase I) or protein kinase A (PKA), as well as phosphorylation of the D domain by CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II), decrease the binding of synapsins to SVs (Hosaka et al., 1999; Huttner et al., 1981; Stefani et al., 1997). Recently, LRRK2 (leucine-rich repeat kinase2) was also found to phosphorylate threonine residues in the C domain and thereby decrease synapsin binding to SVs (Marte et al., 2019). Thus, protein kinases provide a 'phospho-switch' that dynamically controls association of synapsins with SVs (Hosaka et al., 1999).

Self-association of synapsins

In vitro reconstitution experiments show that synapsins bundle and cross-link both actin filaments and microtubules (Bähler & Greengard, 1987; Baines & Bennett, 1986). To explain such results, it was proposed that synapsins 'self-associate'. Subsequent work has proved

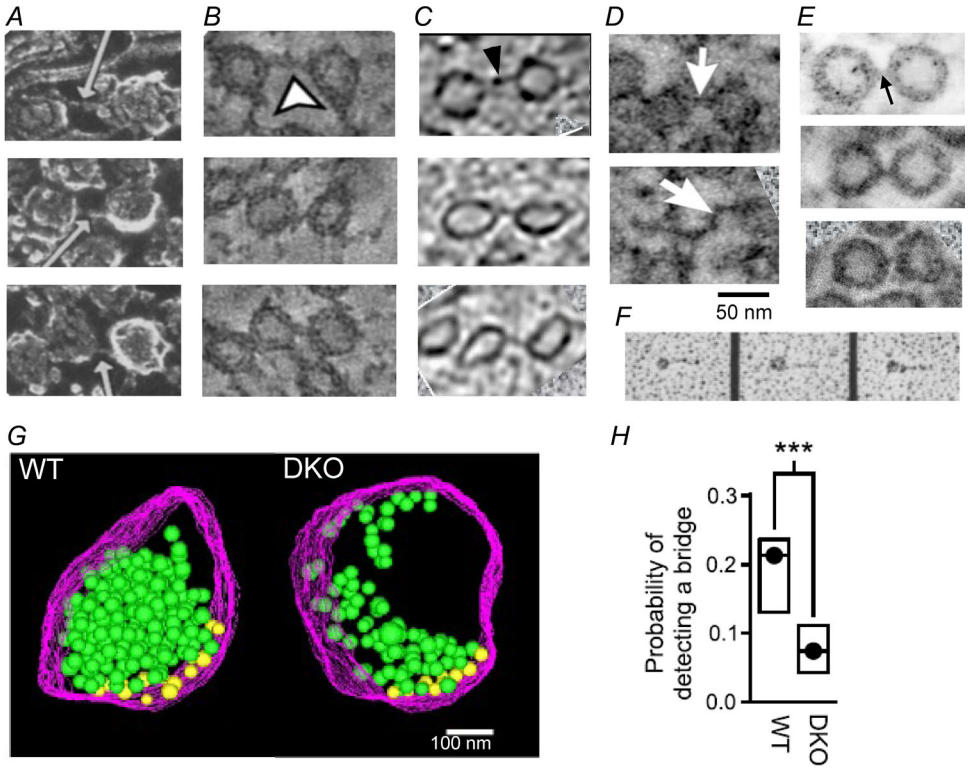

Figure 2. Structure of bridges connecting neighbouring synaptic vesicles
A, grey arrows indicate bridges connecting synaptic vesicles (SVs) in rat cerebellar mossy fibres (from Hirokawa et al., 1989). *B*, white arrowhead indicates bridge between SVs in rat hippocampal slices (from Siksou et al., 2007). *C*, black arrowhead indicates bridge between SVs in rat hippocampal slices (from Fernández-Busnadiego et al., 2010). *D*, white arrows indicate bridges between SVs in rat cultured hippocampal neurons (from Cole et al., 2016). *E*, black arrow indicates a bridge between SVs in mouse hippocampal slice (from Wesseling et al., 2019). *F*, images of the structure of synapsin 1 visualized using low-angle rotary shadowing. The synapsin 1 molecule is ∼50 nm long and tadpole-shaped, with a globular head (∼14 nm in diameter) and a tail that is ∼33 nm long (from Hirokawa et al., 1989). Scale bar in *F* applies to *A*–*F*. *G*, comparison of SVs in synapses from wild-type (WT) and synapsin double knockout (DKO) mice. Volume rendering of electron micrographic tomograph images of presynaptic terminals, with docked SVs represented by yellow spheres and non-docked vesicles represented by green spheres. The magenta lines represent the contours of the presynaptic plasma membrane. *H*, reduction in the number of bridges connecting SVs to their neighbours in DKO mice. Circles are median values, and bars indicate the middle two quartiles of the distributions, ***$p<0.001$ (Kolmogorov-Smirnov test). *G* and *H* are from Wesseling et al. (2019). *D* is reproduced with permission of the authors, while *G* and *H* are reproduced under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

that synapsins do indeed bind to themselves and has established the precise mechanisms involved in such interactions.

Dimerization. Yeast two-hybrid and protein immunoprecipitation measurements show that synapsin isoforms can homodimerize; heterodimers can also form between synapsins 1a and 2a and between synapsins 2a and 3a (Hosaka & Sudhof, 1999). Heterodimerization between synapsin 'a-type' (1a/2a/3a) and 'b-type' (1b/2b) isoforms is less established.

Synapsins bind ATP with high affinity, with half-maximal binding occurring at ATP levels of 0.14–0.45 μM (Hosaka & Sudhof, 1998a,b). Thus, synapsins should be fully bound to ATP in the physiological ATP range (1–2 mM; Rangaraju et al., 2014). ATP has an important influence on synapsin self-association: it enhances homodimerization of both synapsin 1a (Orlando et al., 2014) and synapsin 2a (Song & Augustine, 2023). Such dimerization also occurs in the absence of ATP, indicating that dimerization does not have an absolute requirement for ATP. Surprisingly, Ca^{2+} regulates the ATP-binding properties of synapsins in an isoform-specific way. ATP binding by synapsin 1 requires Ca^{2+} ($EC_{50} = 1.6$ μM), whereas synapsin 2 binds ATP independent of Ca^{2+} (Hosaka & Sudhof, 1998b), and Ca^{2+} inhibits ($IC_{50} = 3.4$ μM) ATP binding by synapsin 3 (Hosaka & Sudhof, 1998a). Commensurate with its effects on ATP binding, Ca^{2+} increases homodimerization of synapsin 1a (Orlando et al., 2014) but not synapsin 2a (Song & Augustine, 2023).

Synapsin dimerization can occur via both covalent and non-covalent bonding. Covalent cross-linking of synapsin 1, via disulphide bonds, occurs in the presence of a cross-linking agent that forms disulphide bonds between neighbouring thiol groups (Font & Aubert-Foucher, 1989). Proteolysis analysis indicates that cysteines in hydrophobic regions of the C domain are responsible for such self-association. Intermolecular disulphide cross-linking of synapsin occurs spontaneously, without any cross-linking reagent, over an extremely slow time scale (1 month; Paranandi & Aswad, 1995). However, such disulphide cross-linking has not been found to occur *in vivo* and thus might not reflect a physiological mechanism for dimerization.

A role for non-covalent, hydrophobic interactions in synapsin dimerization has been established via X-ray

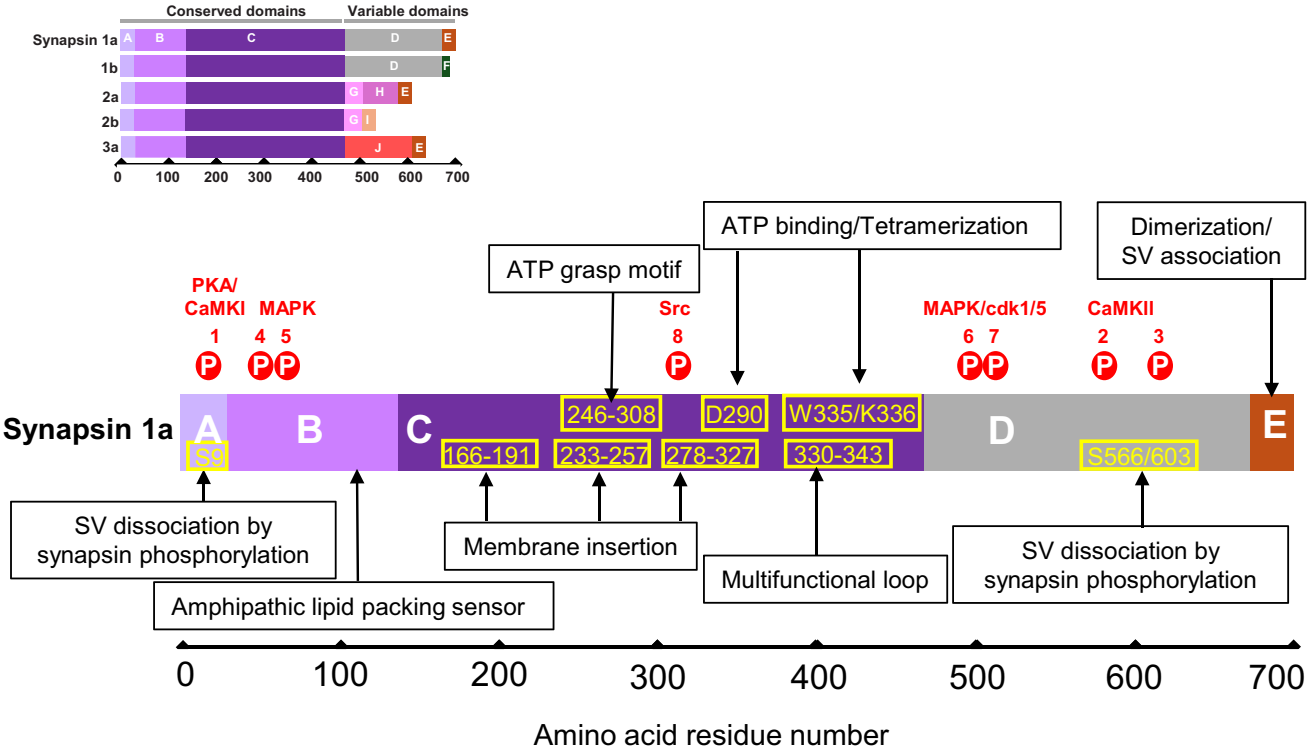

Figure 3. Structure–function relationship of synapsin 1a
Phosphorylation sites and kinases indicated in white rectangles come from experimental measurements on rat synapsin 1a. Amino acid residues in yellow indicate regions involved in synaptic vesicle binding, ATP binding or self-association. The scale at the bottom indicates amino acid positions. The inset shows a diagram of the domain structure of the major synapsin isoforms. Abbreviations: CaMKI (Ca^{2+}/calmodulin-dependent protein kinase I), CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II), cdk (cyclin-dependent kinase), MAPK (mitogen-activated protein kinase), Src (Src family kinase).

crystallographic analysis of the C domain of bovine synapsin 1a (Fig. 4*A*; Esser et al., 1998). The contact surfaces between the two C domains of a synapsin dimer are predominantly hydrophobic, which generates a tight interaction between the two monomers to generate the force for dimerization (Fig. 4*B*). An ATP-binding site, the ATP grasp motif, is located within a central subdomain of the C domain and could be responsible for the ability of ATP to enhance synapsin dimerization (Fig. 4*A*; Esser et al., 1998). Peptides from the synapsin E domain inhibit

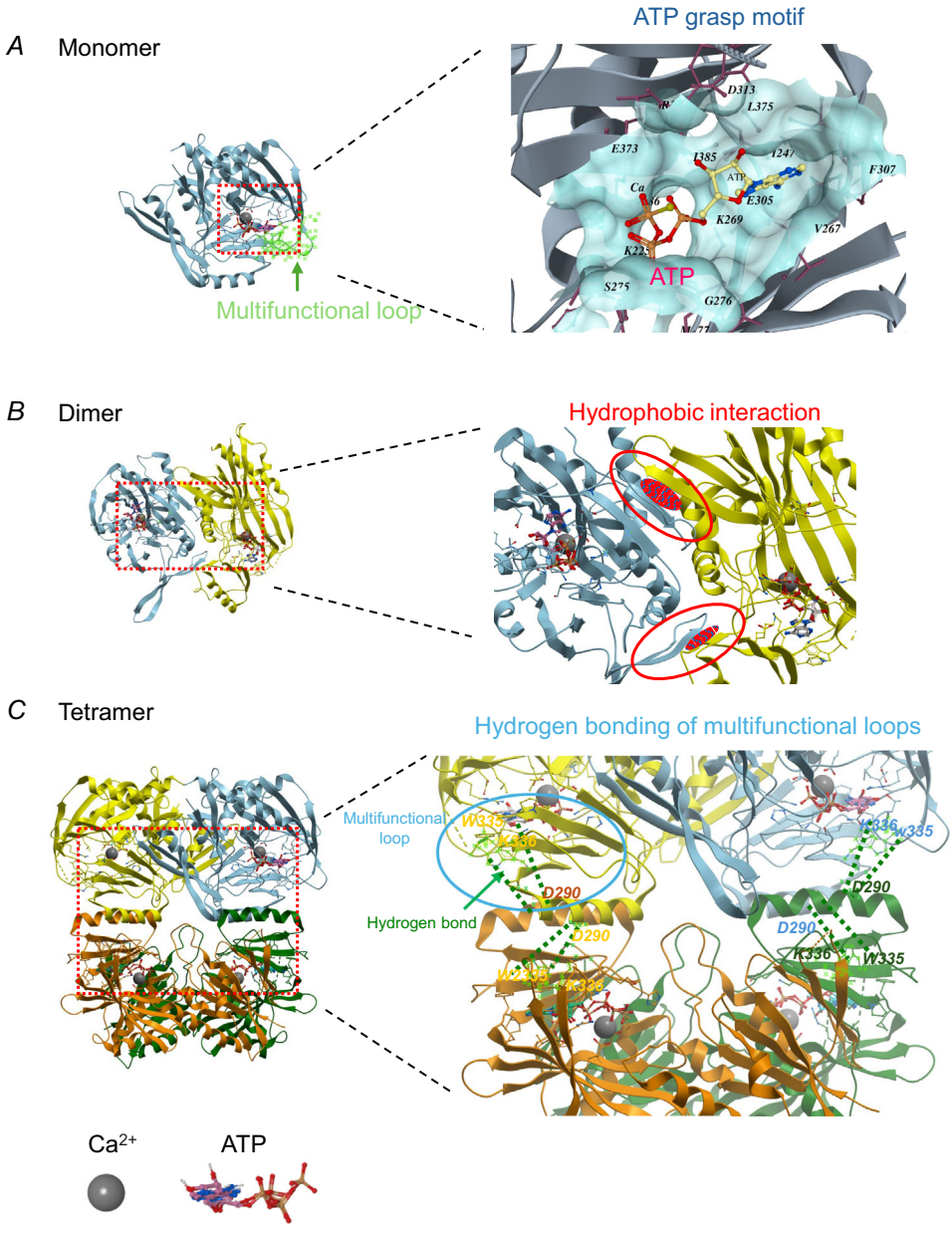

Figure 4. Structures of a monomer (*A*), dimer (*B*) and tetramer (*C*) of the C domain of rat synapsins
A, left, structure of a single C domain. Green indicates the multifunctional loop. *A*, right, magnified view of ATP grasp motif, from the region indicated by the red rectangle in the left image. Labelled residues interact with ATP. *B*, left, structure of dimer consisting of two C domains, indicated in blue and yellow. *B*, right, regions involved in hydrophobic interactions to form dimers, within the region indicated by the red rectangle in the left image, are magnified on the right and are indicated by red ovals and shading. *C*, left, colours indicate four C domains assembled into a tetramer. *C*, right, bonds involved in tetramerization, within the region indicated by the red rectangle in the left image. ATP bound within the ATP grasp motif forms hydrogen bonds with residues W335 and K336 of the multifunctional loop, generating the flexibility to permit an extra hydrogen bond with D290 of the adjacent monomer of another dimer to stabilize the tetramer. The bound ATP molecule is shown in ball-and-stick format, while a grey sphere denotes the position of bound Ca^{2+} ions.

synapsin 1 dimerization; this suggests that the E domain is also involved in synapsin–synapsin interactions, although the structural underpinnings of this interaction have not yet been elucidated (Monaldi et al., 2010).

Tetramerization. Synapsins can also form tetramers. Unlike synapsin dimers, ATP is absolutely required for tetramerization of both synapsin 1a (Orlando et al., 2014) and synapsin 2a (Song & Augustine, 2023). The structure of tetramers of the C domain of synapsin 2a has been determined via X-ray crystallography (Fig. 4*C*; Brautigam et al., 2004). Tetramers are formed by interactions of two dimers, mediated by bonds formed both by the ATP grasp motif and by a multifunctional loop located in the carboxyl portion of the C domain. Specifically, Trp335 and Lys336 make van der Waals contacts with the adenine and ribose of ATP, while also forming hydrogen bonds with the carboxylate moiety of an Asp290 residue of a neighbouring synapsin dimer. These bonds enable ATP-bound dimers to tetramerize, thereby accounting for the ATP requirement of synapsin tetramerization.

Synaptic vesicle clustering by synapsin tetramers

Although self-association of synapsins is well established, only recently has this proporty been shown to be important for synapsin regulation of neurotransmitter release (Orlando et al., 2014; Song & Augustine, 2023). In particular, their tetramerization and SV-binding properties allow synapsins to influence the clustering and mobilization of SVs (Song & Augustine, 2023). *In vitro* reconstitution reveals that synapsin can produce large clusters of SVs (Fig. 5*A*; Song & Augustine, 2023). This effect is attributable to synapsin tetramers,

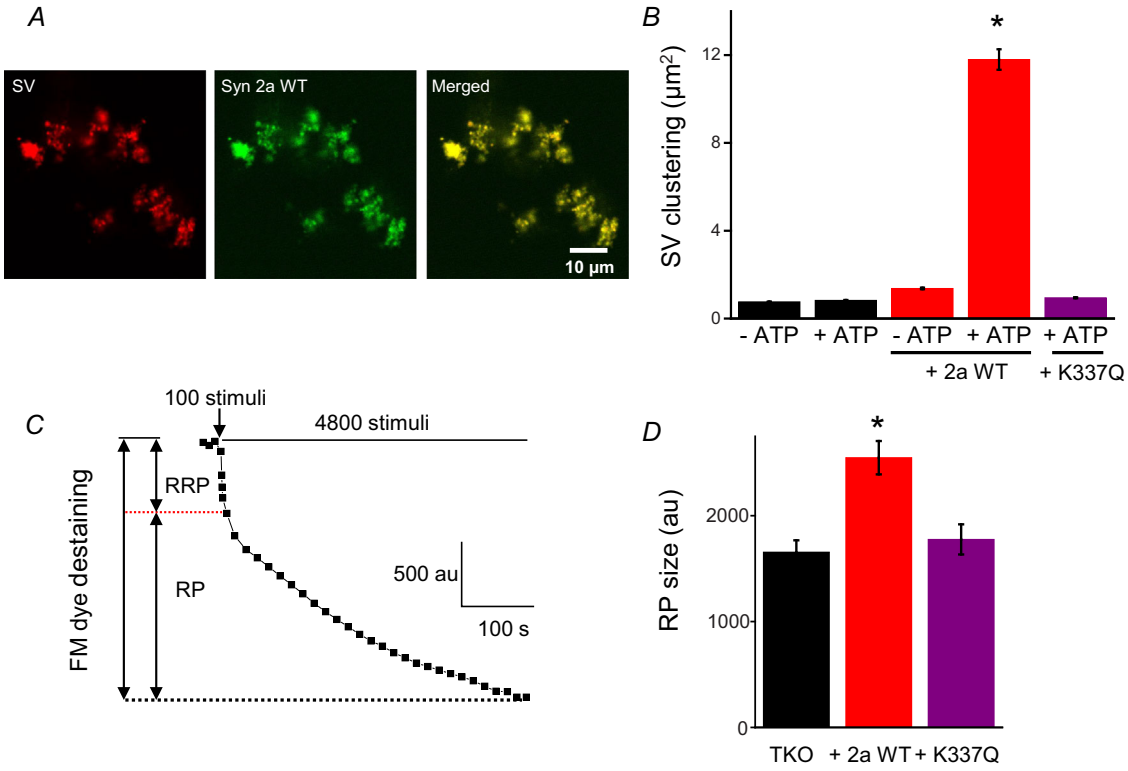

Figure 5. Clustering of synaptic vesicles by ATP-driven synapsin tetramers
A, *in vitro* reconstitution of synaptic vesicle (SV) clustering by combining dye-stained SVs (red; left) with purified green fluorescent protein (GFP)-tagged synapsin 2a (green; middle) in the presence of ATP (5 mM). The merged image (right) shows precise co-localization of SVs and synapsin 2a in large clusters; overlapping pixels are indicated by yellow. *B*, the mean area of SV clusters reconstituted in the experimental conditions at the bottom. Synaptic vesicles cluster very little in the absence of synapsins; they cluster more with wild-type synapsin 2a (2a WT) in the absence of ATP and much more when both synapsin 2a and ATP are present. A tetramerization-deficient mutant form of synapsin 2a (K337Q) does not cluster SVs well, even in the presence of ATP. *C*, FM dye measurement of SV pools. A plot of the kinetics of dye destaining illustrates depletion of the readily releasable pool (RRP) by an initial 100 stimuli, followed by a prolonged loss of dye from the reserve pool (RP) during sustained stimulation (10 Hz). *D*, RP size was determined from FM dye destaining experiments, such as the one shown in *C*, in neurons from synapsin triple knockout (TKO) mice expressing WT synapsin 2a or K337Q. Only WT synapsin 2a rescued the RP of TKO neurons, indicating the importance of synapsin tetramerization for the RP. Statistical comparisons in *B* and *D* were done with ANOVA, followed by Tukey's *post hoc* test; $^*P < 0.05$. From Song and Augustine (2023).

because it requires ATP (Fig. 5*B*) and is absent in a tetramerization-deficient synapsin mutant (K337Q; Fig. 5*B*). Likewise, the tetramerization-deficient mutant does not rescue clustering of SVs in the RP (Fig. 5*C* and *D*) or mobilization of SVs from the RP of glutamatergic hippocampal synapses of synapsin triple knockout (TKO) mice (Song & Augustine, 2023). Furthermore, these results indicate that synapsin dimers are insufficient to cluster SVs, because the tetramerization-deficient mutant dimerizes normally, yet it does not cluster SVs well. Single-molecule tracking of SV mobility shows that K337Q increases the mobility of SVs within the RP (Longfield et al., 2024), consistent with the idea that synapsin tetramers cross-link SVs to form the RP. Collectively, these results strongly suggest that synapsin tetramerization is the mechanism that clusters SVs within the RP at these glutamatergic synapses.

ATP-dependent synapsin tetramerization generates force for SV clustering. ATP produced by mitochondria is required for SV recycling (Pathak et al., 2015). A *Drosophila* mutant (Drp1) lacking mitochondria at their neuromuscular junctions suggests that at least part of this ATP requirement comes from the synapsin-dependent RP. Neuromuscular junctions in these mutants have defects in SV mobilization from the RP, evident as faster synaptic depression during high-frequency stimulation (Verstreken et al., 2005). Similar effects are exhibited by mouse hippocampal neurons expressing a mutant form of synapsin that is defective in ATP binding; these neurons also exhibit faster synaptic depression, which again indicates reduced mobilization of SVs from the RP to the RRP (Shulman et al., 2015). Furthermore, neurons expressing the mutant synapsin deficient in ATP binding have a smaller RP (Song & Augustine, 2023). Thus, both ATP and ATP binding by synapsins influence SV clustering in, and mobilization from, the RP.

Why does clustering of SVs in the RP require synapsin tetramers, rather than dimers? A speculative answer to this question comes from the physiochemical properties of SV membranes, which contain the negatively charged phospholipid, phosphatidylserine (Binotti et al., 2021; Ohsawa et al., 1981). Such negative charges will create short-range repulsive forces that drive SVs apart. Measurements made on artificial membranes indicate that electrostatic repulsion between charged membranes drops exponentially with distance, with a length constant of 1.2 nm for a membrane composed of phosphatidylserine (Pera et al., 2004). As a result, SV clustering by synapsins requires an interaction that can interconnect neighbouring SVs while avoiding local repulsive forces that act over a range of ∼7 nm (three length constants for each membrane). The globular C domain in the head of synapsins, which mediates tetramerization, has a large size that can generate the required spacing between two SVs. A dimer of synapsin C domains has a longest dimension of 6.8 nm, while for tetramers it is 8.0 nm (Fig. 6*A*; Brautigam et al., 2004). Although the structural contributions of the tail of synapsins are unclear, these dimensions make clear that the larger synapsin tetramer is better for overcoming electrostatic repulsion. Furthermore, tetramers provide a more stable attachment to SVs by binding two synapsins, rather than one, to each SV (Fig. 6*Bi* and *Bii*). Thus, we propose that synapsin oligomers serve both as a spacer that circumvents short-range electrostatic repulsion between SVs and as a long-range linker that interconnects SVs to form the RP.

One obvious problem with the tetramer-bridging model shown in Fig. 6*Bii* is that the length of this proposed cross-linker is ∼8 nm, shorter than the length of most of the bridges shown in Fig. 2*A*–*E*. This suggests that the bridges between SVs might be formed by synapsin oligomers larger than tetramers (i.e. multiple tetramers). A synapsin octomer (Fig. 6*Biii*) would make a structure (https://www.ebi.ac.uk/pdbe/entry/pdb/1pk8) that more closely resembles the inter-SV bridges shown in Fig. 2*A*–*E*. Formation of such higher-order structures could be mediated by the same type of hydrogen bonds that connect pairs of dimers in the tetrameric structure shown in Fig. 4*C*; by spanning across tetramers, these bonds could assemble structures similar in size to the SV bridges. The unstructured tails of synapsins could extend the length of the bridges further. Alternatively, the SV bridges could include proteins in addition to synapsin tetramers. For example, recent studies indicate that α-synuclein binds to synapsins and that perturbing this interaction disrupts SV clustering (Fouke et al., 2021; Stavsky et al., 2023). In summary, although clustering of glutamatergic SVs requires synapsin tetramers, and tetramers fulfil the requirements to serve as bridges to cross-link SVs, the precise relationship between tetramers and SV bridges is not yet clear.

Alternative models for synapsin-mediated SV clustering

The sections above summarize the oligomerization properties of synapsins and the recent evidence indicating that synapsin tetramers are responsible for cross-linking SVs within the RP of glutamatergic synapses. However, alternative models have been proposed for clustering of SVs, and we conclude by briefly considering these alternatives.

A long-standing model proposes that binding of synapsins to actin filaments plays a crucial role in maintaining SVs within the distal RP cluster. According to this model, synapsins bind to both actin filaments and SVs, effectively tethering SVs to the actin

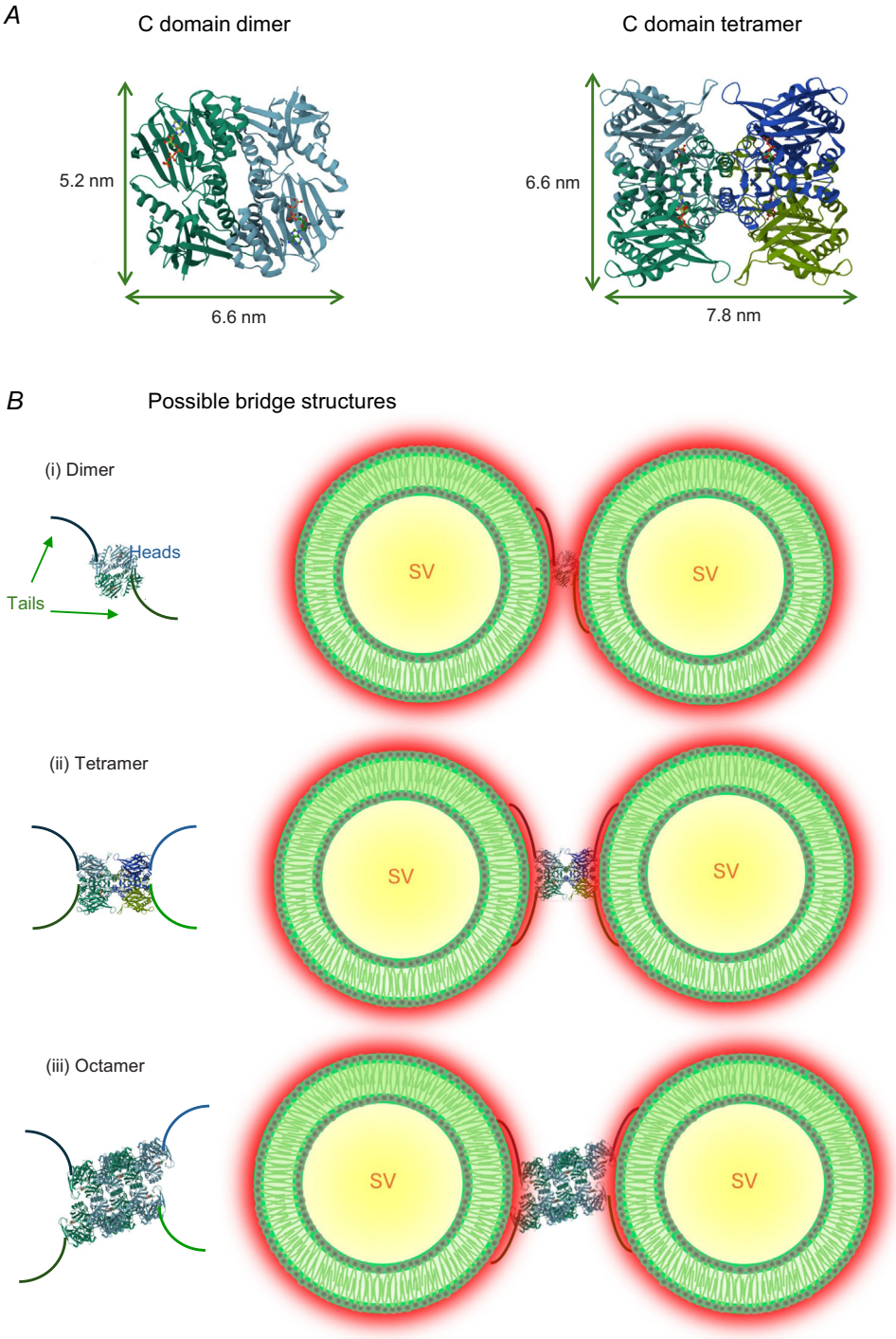

Figure 6. Models of synapsin-dependent bridges between synaptic vesicles
A, structures of a dimer (left) and a tetramer (right) of rat synapsin 2a C domains, viewed from the crystallographic 2-fold axis. Synapsin monomers are individually coloured and are shown on the same spatial scale; dimensions were determined from the protein data bank (https://doi.org/10.2210/pdb1PX2/pdb). *B*, comparison of potential synaptic vesicle (SV) cross-links formed by synapsin oligomers. Left, structures of indicated synapsin oligomers. Right: (*i*) the separation provided by a synapsin dimer is too short to avoid electrostatic repulsion, produced by negative charge (red) associated with acidic lipids in the SV membrane, between neighbouring SVs (green). However, both synapsin tetramers (*ii*) and octamers (*iii*) can separate neighbouring SVs sufficiently to avoid such electrostatic repulsion.

cytoskeleton and thereby clustering SVs (Greengard et al., 1993). Experimental evidence supports the ability of synapsins to bind to actin and even to promote actin polymerization and bundling of actin filaments (Bähler & Greengard, 1987; Benfenati et al., 2004; Hirokawa et al., 1989). However, counterarguments suggest that this model is incorrect. First, depolymerizing actin filaments does not disperse the RP cluster (Bloom et al., 2003; Sankaranarayanan et al., 2003). Second, there is a lack of actin-like filaments associated with the SV cluster (Fernandez-Busnadiego et al., 2010). Instead, actin filaments are positioned prominently around the periphery of the active zone, where they are likely to contribute to SV endocytosis/recycling (Shupliakov et al., 2002). Thus, it appears that actin influences the RP only indirectly, by participating in endocytosis, which recycles SVs following exocytosis. In summary, the actin scaffold model of SV clustering has fallen out of favour (Zhang & Augustine, 2021).

As mentioned in the Introduction, synapsins undergo LLPS (Alfken et al., 2024; Hoffmann et al., 2023; Milovanovic et al., 2018; Pechstein et al., 2020; Song & Augustine, 2023). LLPS is a dynamic process that contributes to synapse development and synaptic signalling and potentially plays a role in psychiatric disorders (Chen et al., 2020). It has been proposed that LLPS could also play a role in clustering of SVs by synapsins (Milovanovic et al., 2018; Pechstein et al., 2020; Qiu et al., 2024; Song & Augustine, 2023). Consistent with this model, LLPS by synapsins can cluster liposomes (Alfken et al., 2024; Hoffmann et al., 2023; Milovanovic et al., 2018; Qiu et al., 2024) and can mildly cluster SVs *in vitro* (Hoffmann et al., 2023; Qiu et al., 2024; Song & Augustine, 2023). However, synapsin 1a, the isoform best at generating LLPS (Milovanovic et al., 2018; Song & Augustine, 2023), does not rescue the glutamatergic RP of hippocampal neurons from synapsin TKO mice (Gitler et al., 2008). Furthermore, interfering with synapsin LLPS does not affect mobilization of SVs from the RP of these synapses (Song & Augustine, 2023). This suggests that synapsin LLPS is not required for clustering of SVs within the RP of hippocampal glutamatergic synapses. In addition, conditions that promote synapsin LLPS do not produce cross-bridges between SVs *in vitro* (Alfken et al., 2024), unlike the condition *in vivo* (see Fig. 2).

Synapsin LLPS does play a role in SV clustering at hippocampal GABAergic synapses. At these inhibitory synapses, a tetramerization-deficient synapsin mutant is equivalent to WT synapsin in rescuing the RP phenotype of synapsin TKO mice (Song & Augustine, 2023). Furthermore, this mutant also rescues the synchronization of GABA release that is impaired in TKO neurons, while disrupting LLPS desynchronizes GABA release in neurons expressing WT synapsins (Song & Augustine, 2023). There are additional suggestions that synapsin LLPS might contribute to clustering of SVs at the giant reticulospinal synapse of lampreys (Pechstein et al., 2020). Therefore, it appears that both LLPS and tetramerization of synapsins can cluster SVs within presynaptic terminals, with the relative contributions of each mechanism varying between synapses.

Conclusions

Although a very substantial body of evidence supports a role for synapsins in maintaining SVs within the distal RP cluster of glutamatergic synapses (Hilfiker et al., 1999; Zhang & Augustine, 2021), the precise mechanism by which they achieve this has been unresolved and somewhat controversial. It has long been known that synapsins can oligomerize (Brautigam et al., 2004; Hosaka & Sudhof, 1998b; Orlando et al., 2014; Song & Augustine, 2023), though the physiological role of such self-association has remained unclear until very recently. Evidence described here presents coherent support for the idea that synapsin tetramerization is required for clustering of glutamatergic SVs within the RP of hippocampal neurons (Song & Augustine, 2023). Glutamatergic synapses have larger RPs than those of GABAergic inhibitory synapses; therefore, synapsin tetramerization might provide a more efficient mechanism for clustering SVs in comparison to clustering via LLPS. This is consistent with *in vitro* reconstitution experiments showing that synapsin tetramerization clusters SVs more effectively than LLPS (Song & Augustine, 2023).

Given that synapsin tetramers cross-link glutamatergic SVs, the next step will be to determine how these SVs are mobilized from the RP during synaptic activity. Extensive experimental evidence indicating that protein kinases provide a 'phospho-switch' that dynamically controls association of synapsins with SVs provides a possible mechanism for freeing SVs from their clusters during mobilization (Greengard et al., 1993; Hosaka et al., 1999). Future work must consider the influence of phosphorylation on synapsin-dependent cross-linking of glutamatergic SVs, in addition to other proposed mechanisms of mobilization of these SVs from the RP (Qiu et al., 2024).

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Additional information

Competing interests

None declared.

Author contributions

S.-H.S. and G.J.A. developed the figures and drafted the manuscript. Both authors approved the final version of the manuscript. Both people designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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