Vesicle Clustering in a Living Synapse Depends on a Synapsin Region that Mediates Phase Separation


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Vesicle Clustering in a Living Synapse Depends on a Synapsin Region that Mediates Phase Separation

Graphical Abstract

Highlights

- Reagents that bind to the IDR of synapsin cause dispersal of synaptic vesicle clusters
- Inhibition of SH3 domain interactions with synapsin do not cause vesicle dispersal
- Synaptic vesicle clustering \textit{in vivo} can be explained by phase separation

Authors

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In Brief

Pechstein et al. report that presynaptic microinjection of reagents that bind to the intrinsically disordered region (IDR) of synapsin cause dispersal of synaptic vesicle clusters. Inhibition of SH3 domain interactions with synapsin do not cause vesicle dispersal. Synaptic vesicle clustering \textit{in vivo} can be explained by liquid-liquid phase separation.
Vesicle Clustering in a Living Synapse Depends on a Synapsin Region that Mediates Phase Separation

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SUMMARY

Liquid-liquid phase separation is an increasingly recognized mechanism for compartmentalization in cells. Recent in vitro studies suggest that this organizational principle may apply to synaptic vesicle clusters. Here we test this possibility by performing microinjections at the living lamprey giant reticulospinal synapse. Axons are maintained at rest to examine whether reagents introduced into the cytosol enter a putative liquid phase to disrupt critical protein-protein interactions. Compounds that perturb the intrinsically disordered region of synapsin, which is critical for liquid phase organization in vitro, cause dispersion of synaptic vesicles from resting clusters. Reagents that perturb SH3 domain interactions with synapsin are ineffective at rest. Our results indicate that synaptic vesicles at a living central synapse are organized as a distinct liquid phase maintained by interactions via the intrinsically disordered region of synapsin.

INTRODUCTION

Chemical synapses can sustain neurotransmitter release at high rates by mobilizing synaptic vesicles (SVs) from a pool clustered at the release sites. Despite the critical role of SV clusters in neural signaling, the mechanisms underlying their organization remain unclear. Two principal models have been proposed, both of which are centered at synapsins that are essential for the maintenance of the major, distal part of SV clusters (Pieribone et al., 1995; Rosahl et al., 1995). In the first model, called the scaffold model here, the clustering of SVs is suggested to depend on their anchoring via synapsins to the cytoskeleton, because synapsins can bind both SVs and cytoskeletal components like actin, spectrin, and microtubules (Cingolani and Goda, 2008; Greengard et al., 1993; Hilfiker et al., 2005). Key to the scaffold model is the observation that Ca²⁺-dependent phosphorylation of synapsin by Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) causes its dissociation from SVs and actin, thereby enabling vesicle mobilization (Greengard et al., 1993). A variant of the scaffold model implies that SVs are kept in place by intervesicular filaments, or tethers, that are partly composed of synapsin but also contain unidentified components (Shupliakov et al., 2011; Siksou et al., 2011; Zuber and Lučić, 2019). In the second model, called the liquid phase model here, SV clustering is suggested to result from phase separation (Milovanovic and De Camilli, 2017). Liquid-liquid phase separation is an increasingly recognized mechanism for membraneless subcellular compartmentalization, in which a distinct liquid phase forms in the cytosol because of weak multimeric interactions between proteins or between RNA and proteins (Banani et al., 2017; Gomes and Shorter, 2019). In the case of protein-based liquid phase organization, the interactions typically involve intrinsically disordered regions (IDRs) and/or modular binding domains such as SH2 and SH3 domains (Banani et al., 2017; Gomes and Shorter, 2019). In support of the liquid phase model for SV clustering, in vitro studies have shown that recombinant synapsin, or its isolated IDR (also termed region D), can form droplets in aqueous solution (Milovanovic et al., 2018). Droplet formation was enhanced by adding synapsin-binding SH3 domains and disrupted by CaMKII phosphorylation. Moreover, small liposomes could be captured in synapsin droplets (Milovanovic et al., 2018). At present, neither of the two models of SV clustering has been rigorously tested in vivo. Moreover, with regard to liquid-liquid phase separation, in vitro results are not easily transferred to a cellular context (Alberti et al., 2019).

Here we examine whether the requirements for synapsin droplet formation in vitro pertain to SV clusters at the lamprey giant reticulospinal synapse. Earlier studies in this model have demonstrated that SV clusters in stimulated synapses depend on synapsin (Pieribone et al., 1995). In the present experiments, we maintained synapses at rest to test whether reagents microinjected into the axonal cytosol would enter a putative vesicular liquid phase to disrupt critical protein-protein interactions. We found that compounds that perturb the synapsin IDR cause dispersion of SVs from resting clusters. However, reagents that perturb SH3 domain interactions with synapsin are ineffective.
RESULTS

Antibodies Binding the Synapsin IDR Disrupt SV Clusters Maintained at Rest

A prediction of the liquid phase model is that compounds injected into the axonal cytosol will exchange with, and move freely within, resting SV clusters (Bergeron-Sandoval et al., 2016; Brangwynne et al., 2009). Microinjection into resting axons of compounds targeting the synapsin IDR (Milovanovic et al., 2018) could thus give evidence for the liquid phase model in vivo. The turnover of SVs at this synapse at rest is low (Shupliakov et al., 1997) implying that the resting cluster is stable. Moreover, the index of disorder of the lamprey synapsin sequence (Kao et al., 1999) is virtually identical to that of mammalian synapsin (Ishida and Kinoshita, 2007; Milovanovic et al., 2018), and the IDR of lamprey synapsin readily formed droplets in vitro (Figure 1).

Axons microinjected with an inactive control antibody, as well as un.injected axons, contained large, densely packed SV clusters (Figures 2A and 2D). In contrast, in axons microinjected with antibodies raised and immunopurified against the synapsin IDR, the distal part of the SV cluster was lost (Figures 2B and 2D). Only a few layers of vesicles remained tethered at the active zone. Notably, numerous single SVs and small SV aggregates were scattered in the cytosol (arrows in Figure 2B; Figure S1). Dense condensates occurred in association with vesicles (Figure S1C). At synapses exposed to lower antibody concentrations (see STAR Methods), partly disrupted vesicle clusters with vesicle aggregates associated with dense condensates of different sizes were observed (Figure S1D). The loss of the distal vesicle cluster shown in Figure 2B thus appears to be the end stage of a process of dissociation of previously clustered vesicles. Endocytic clathrin-coated intermediates were rarely observed, consistent with the resting state of synapses. The antibodies were also tested on droplet formation of the synapsin IDR in vitro. We found that the IDR antibodies stimulated droplet formation (Figure 3), consistent with condensate formation by the divalent antibodies and the IDR.

Under the same conditions, the E region-directed antibodies that were used in our previous study of stimulated synapses (Pieribone et al., 1995) had only a partial effect on resting vesicle clusters (Figures 2C and 2D). The reduction in the number of SVs was small and not significant in comparison with synapses in control axons. Some individual dispersed SVs occurred outside clusters, but disruption of clusters into SV aggregates (cf. Figures S1C and S1D) was not observed. Reanalysis of our previous material (Pieribone et al., 1995) confirmed that the E
region-directed antibodies only caused significant loss of SVs in synapses that were active following microinjection.

We conclude that antibodies directed to the IDR of synapsin enter and disrupt SV clusters in resting axons as predicted by the liquid phase model.

**A Fusion Protein Binding the IDR of Synapsin Disrupts SV Clusters Maintained at Rest**

The SH3 domain-containing region of intersectin increases the size of synapsin droplets in vitro but disrupts them when applied in excess, presumably by interfering with IDR-IDR interactions (Milovanovic et al., 2018). Hence, we used an intersectin SH3 domain as an alternative way to target the synapsin IDR. The SH3A domain was selected, because it effectively binds synapsin, whereas the other four SH3 domains of intersectin did not show detectable binding (Figure 4A). The binding site of the SH3A domain was mapped to a proline-rich stretch within the synapsin IDR (Figure 4B; Figure S2).

SV clusters in control axons injected with control glutathione S-transferase (GST) protein, similar to uninjected axons, were large and densely packed (Figures 4C and 4E). In axons microinjected with the SH3A domain fused with GST, the distal part of the SV cluster was lost, with only a few layers of vesicles tethered at the active zone (Figures 4D and 4E). Numerous single vesicles were scattered in the cytosol, along with aggregates of SVs (Figure 4D; Figure S3). The effect of the SH3A domain was also tested on droplet formation of the synapsin IDR in vitro. We found that the SH3A domain potently inhibited droplet formation (Figure 5). Additional in vitro experiments confirmed that the SH3A domain interacts with synapsin when it is bound to lipid vesicles (Figure S4).

In additional control experiments, axons were microinjected with the SH3B domain of intersectin fused with GST. This domain binds endophilin but does not bind synapsin at detectable levels (Pechstein et al., 2015; Figure 4A). These axons contained large, densely packed vesicle clusters (Figure 4E; Figure S3D).

We conclude that an SH3 domain that interacts with the synapsin IDR enters and disrupts SV clusters in agreement with the liquid phase model.

**Antibodies Blocking the Intersectin SH3A-Synapsin Interaction Do Not Disrupt SV Clusters Maintained at Rest**

IDR-IDR interactions and SH3 domain interactions may contribute to droplet formation of synapsin in vitro (Milovanovic et al., 2018). The synapsin IDR-directed antibodies and the SH3A domain are expected to simultaneously perturb both in living synapses and thus do not allow conclusions about which of these interaction types is essential in vivo.

To examine the role of the intersectin SH3A-synapsin interaction, we prepared antibodies directed to the SH3A domain. Competition assays showed that these antibodies inhibited the interaction of the SH3A domain with synapsin (Figure 6A). Microinjected SH3A-directed antibodies had no effect on resting SV clusters (Figures 6B and 6E), indicating that the intersectin-synapsin interaction is not essential for maintenance of SV clusters at rest.

In addition to intersectin, other SH3 domain-containing proteins, including amphiphysin, endophilin, and syndapin, are enriched in SV clusters (Andersson et al., 2008; Bai et al., 2010; Evergren et al., 2004; Sundborger et al., 2011). Our binding experiments (Figure 6C) showed that the SH3 domains of only intersectin and amphiphysin bound synapsin effectively, whereas those of endophilin and syndapin bound synapsin weakly. Moreover, our previous studies have shown that disruption of SH3 domain interactions of the latter two proteins does not cause disruption of SV clusters (Andersson et al., 2008; Gad et al., 2000). Hence, we prepared antibodies to the SH3 domain...
of amphiphysin. When microinjected, these antibodies did not disrupt SV clusters under resting conditions (Figures 6D and 6E). Together with previous results, the present data thus indicate that SH3 domain-containing proteins are not essential to maintain SV clusters at rest.

**Action Potential Stimulation Reveals Biological Activities of Intersectin SH3A Antibodies and Amphiphysin SH3 Antibodies**

In the experiments with resting axons described earlier, neither the intersectin SH3A domain antibodies nor the amphiphysin SH3 domain antibodies produced detectable effects on vesicle clusters. To rule out the possibility that these reagents were biologically inactive, we tested their efficacy during synaptic activity.

Both reagents had evident effects when action potential stimulation at 5 Hz was applied after the microinjection (Figure 7). The intersectin SH3A antibodies induced a significant increase in the number of clathrin-coated pits in the periactive zone, along with a reduction in the number of SVs (Figures 7A and 7C), likely because of perturbation of the interaction with dynamin (Pechstein et al., 2010b). Similarly, the amphiphysin SH3 antibodies caused an increase in the number of clathrin-coated pits at the periactive zone (Figures 7B and 7C), again presumably because of disruption of the interaction with dynamin (Shupliakov et al., 1997).

**DISCUSSION**

Recent studies with purified components suggest that presynaptic compartments, including the SV cluster and the active zone, may be organized by liquid-liquid phase separation (Milo-vanovic et al., 2018; Wu et al., 2019). However, the precise biological relevance of these data has been unclear (Alberti et al., 2019). Here we used a living giant synapse to examine the conditions underlying organization of the SV cluster. Our results fulfill two main criteria predicted by the liquid phase model. First, fusion proteins and antibodies microinjected into the axonal cytosol readily entered, and affected, key interaction sites within resting SV clusters containing thousands of densely packed vesicles (Gustafsson et al., 2002). This finding is in line with the property of a liquid phase compartment (Bergeron-Sandoval et al., 2020). The GST-SH3B fusion protein, which was also without effect under resting conditions (Figure 4E), caused a modest reduction in the number of SVs, along with the occurrence of clathrin-coated pits and free clathrin-coated vesicles, in agreement with previous data (Pechstein et al., 2015). Free coated vesicles are almost never seen in control axons, and their occurrence likely results from interference with the interaction between intersectin and endophilin, which participates in uncoating via synaptojanin (Gad et al., 2000; Pechstein et al., 2015).

We conclude that the intersectin SH3A antibodies, the amphiphysin SH3 antibodies, and the GST-SH3B fusion protein are all biologically active, as proven by their effects on endocytic protein-protein interactions uncovered by synaptic activity. Further proof of the effect of synaptic activity was obtained by applying stimulation after microinjection of the GST-SH3A fusion protein (cf. the resting condition in Figure 4D). Under these conditions, this reagent not only caused loss of the SV cluster but also inhibited endocytosis, as shown by the occurrence of numerous constricted and unconstricted clathrin-coated pits (Figure S5), in agreement with earlier studies in non-neuronal cells (Simpson et al., 1999).
The low level of spontaneous vesicle turnover in the lamprey synapse (described earlier) underscores that the SV cluster can be regarded as near a complete resting state. If the cluster had been organized as a rigid scaffold structure, it would have been expected that the reagents penetrated less effectively, thus not giving rise to complete dispersion of clustered vesicles. Second, and most importantly, the IDR defined as critical for synapsin droplet formation in vitro was found to be equally critical for maintaining SV clusters in living synapses. Disturbance of the IDR, by binding of antibodies or the SH3A domain, led to complete dissociation of the distal part of SV clusters. Our in vitro experiments confirmed the efficacy of the reagents used.

The SH3A domain potently inhibited droplet formation of the IDR. Its efficacy may suggest that the region within the lamprey IDR that contains the SH3A binding site plays an important role to support IDR-IDR interactions. In mammalian synapsin I, at least two SH3A binding sites have been identified (Gerth et al., 2017). Thus, it cannot be excluded that occlusion of several SH3A domain binding sites may be required to disrupt liquid droplet formation. Higher than stoichiometric amounts of SH3A domains are more efficient to disperse synapsin IDR phase separation. Higher than stoichiometric amounts of SH3A domain binding sites may be required to disrupt liquid droplets.

Although the present results do not formally disprove the scaffold model, several arguments can be raised against it. The original version of the scaffold model implies that SVs are anchored via actin filaments (Cingolani and Goda, 2008; Greengard et al., 1993; Hilfiker et al., 2005). This possibility appears unlikely for two reasons. First, it would suggest that the C and E regions, which mediate actin binding (Hilfiker et al., 2005), would be important, rather than the IDR. Second, actin filaments are mainly localized around SV clusters, with few filaments penetrating their interior (Bloom et al., 2003; Siksou et al., 2011). The version of the scaffold model suggesting a role for intervesicular tethers is more difficult to rule out, because their molecular identity is unclear, with only part consisting of synapsin. However, the estimated number of synapsin molecules per vesicle does not correlate well with the number of tethers (Wang and Kaeser, 2018). Moreover, intervesicular tethers can still be observed after knockout of synapsin (Siksou et al., 2007), which argues against their primary role in SV clustering.

It is important to note that the present results only concern the resting synapse, and the situation may be somewhat different during activity. Stimulation causes a fraction of the vesicular synapsin pool to disperse into the axon, at least partly because of its phosphorylation by CaMKII (Chi et al., 2001). It is conceivable, and consistent with in vitro data (Milovanovic et al., 2018), that the mobile synapsin fraction temporarily leaves the liquid phase to reenter it during rest.

We found that the antibodies directed to region E of synapsin did not effectively disrupt vesicle clusters at rest. In our earlier experiments, these antibodies were shown to deplete the distal vesicle pool when the microinjection was followed by action potential stimulation (Pieribone et al., 1995). This stimulus-dependent effect can be explained by the stimulation-induced

![Figure 4. A Fusion Protein Binding the IDR of Synapsin Disrupts SV Clusters Maintained at Rest](image-url)
The dispersal of synapsin discussed earlier. Following its dispersal in the cytosol, synapsin may have been captured by the E region-directed antibodies and thus prevented from reassociating with SVs to maintain a liquid phase. It is possible that the marked stimulus-dependent effect is enhanced by exposure of a normally hidden E region. The E region-directed antibodies produced a minor, non-significant loss of vesicles. The lack of a consistent effect suggests that the E region is not involved in maintaining the liquid phase. We speculate that the modest effect results from mobility of some vesicles even at rest that may be linked with minute dispersion of synapsin. It is also possible that the E region-directed antibodies exerted a small influence on the adjacent IDR within a liquid phase.

Another notable observation was the occurrence of numerous scattered SVs and SV aggregates in the axonal cytosol following IDR perturbation, which sheds new light on a long-standing problem. Knockout of synapsins is known to cause a general decrease in the number of SVs, which has led to the proposition that synapsins play a role in SV maintenance, possibly via its ATP-binding C region (Rosahl et al., 1995; Sudhof, 2004). In an alternative hypothesis, the loss of SVs was proposed to be secondary to a clustering defect, eventually resulting in SV degradation (De Camilli, 1995). The dispersion of SVs following acute IDR perturbation supports a primary role of synapsin in vesicle clustering in central synapses.

A proximal pool of SVs remained tethered at the active zone after IDR perturbation. The mechanisms underlying the organization of this membrane-proximal pool are presently unclear. It may be organized by scaffolding, with the vesicles tethered to filaments extending from the active zone (Gundelfinger et al., 2016; Siksou et al., 2011; Wang and Kaeser, 2018). Alternatively, these vesicles may compose a distinct liquid phase organized by other proteins than synapsin. The active zone has been proposed to form a distinct liquid phase involving RIM, RIM-BP, and calcium channels (Wu et al., 2019), but it is unclear how far into the cytosol this phase may extend.

Intersectin, amphiphysin, endophilin, and syndapin are all localized in SV clusters at rest. Upon stimulation, they partly redistribute to the periactive zone, where they participate in different aspects of SV endocytosis (Andersson et al., 2008; Bai et al., 2010; Evergren et al., 2004, 2007; Sundborger et al., 2011). Although the endocytic functions of these proteins are well established, their possible roles within the SV cluster remain unclear. We did not observe any effect on resting SV clusters by disrupting intersectin or amphiphysin SH3 domain interactions with synapsin, which is consistent with results on intersectin- and amphiphysin-deficient mice (Di Paolo et al., 2002; Gerth et al., 2017). In our previous studies, we did not observe any effect on clusters after perturbing endophilin or syndapin SH3 domain interactions (Andersson et al., 2008; Gad et al., 2000). Thus, individual interactions with any one of these four presynaptic SH3 domain-containing proteins are dispensable for the maintenance of resting SV clusters in vivo. However, we do not rule out the possibility that multivalent interactions...
simultaneously involving these proteins may promote liquid phase organization of the SV cluster.

The accumulation of SH3 domain-containing proteins in SV clusters may merely reflect passive buffering (Bai et al., 2010; Denker et al., 2011), but they may also play active roles at this location (Pechstein and Shupliakov, 2010; Shupliakov, 2009). In the case of intersectin, a role in SV mobilization has been proposed, because deletion of intersectins I and II in mice causes enhanced synaptic depression during high-frequency stimulation (Gerth et al., 2017). Such enhanced depression is consistent

Figure 6. Antibodies Blocking SH3 Domain Interactions Do Not Disrupt SV Clusters Maintained at Rest

(A) Antibodies to the SH3A domain of intersectin (ITSN) inhibit its binding with synapsin and dynamin in lamprey brain extract. The panels show immunoblots with antibodies to dynamin, synapsin, and β-actin.

(B) Electron micrograph of a reticulospinal synapse in a resting axon microinjected with antibodies to the SH3A domain of lamprey intersectin.

(C) Binding of proteins in lamprey brain extract to GST fusion proteins with SH3 domains from intersectin (SH3A), amphiphysin, endophilin, and syndapin, respectively. The panels show blots with antibodies to synaptojanin, dynamin, and synapsin. β-actin antibodies were used as control.

(D) Synapse in a resting axon microinjected with antibodies to the SH3 domain of lamprey amphiphysin.

(E) Quantification of the number of SVs in synapses of un.injected resting control axons, under the conditions shown in (B) and (D), and in synapses from axons microinjected with control IgG. n of synapses for ctrl, 10 (3 axons); ITSN1 SH3A Ab, 11 (3 axons); Amphiphysin (Amph) SH3 Ab, 8 (2 axons); and IgG, 15 (3 axons).

Designations in electron micrographs as in Figure 1. Scale bar in (D) represents 0.5 μm for (B) and (D).

Figure 7. Action Potential Stimulation Reveals Biological Activities of Antibodies to SH3 Domains of Intersectin and Amphiphysin

(A) Electron micrograph of the periactive zone (2 μm from the edge of the active zone) in a reticulospinal synapse in an axon stimulated at 5 Hz for 20 min following microinjection of antibodies to the SH3A domain of lamprey intersectin.

(B) Periactive zone in a synapse in an axon stimulated at 5 Hz following microinjection of antibodies to the SH3 domain of lamprey amphiphysin.

(C) Quantification of the number of clathrin-coated pits in synapses from uninjectected control axons stimulated at 5 Hz and under the conditions shown in (A) and (B). n of synaptic periactive zones for ctrl, 20 (3 axons); ITSN1 SH3A Ab, 20 (3 axons); and Amph SH3 Ab, 20 (3 axons). Mean ± SD; ***P < 0.001.

(D) Synapse in an axon stimulated at 5 Hz following microinjection of the SH3B domain of lamprey intersectin fused with GST. Note the occurrence of free clathrin-coated vesicles (ccv).

Scale bar in (B) represents 0.5 μm for (A) and (B) and 0.2 μm for (D).
with impaired SV mobilization, but it could also be secondary to an endocytosis defect compromising the clearance of release sites (Hua et al., 2013; Shupliakov et al., 1997). The roles of SH3 domain-containing proteins in SV clusters thus remain an important topic for future studies.

STAR+METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.01.092.

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AUTHOR CONTRIBUTIONS

A.P., L.B., and O.S. designed experiments; A.P., L.B., O.S., and O.V. performed biochemical experiments; K.F., A.P., and O.S. generated key reagents for the study; L.B., N.T., E.S., E.E., and O.S. performed microinjections and electron microscopy; A.P., N.T., L.B., and O.S. analyzed data; and L.B. and O.S. wrote the manuscript.

DECLARATION OF INTERESTS

Authors declare no competing interests.

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KEY RESOURCES TABLE

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LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Oleg Shupliakov (oleg.shupliakov@ki.se).

All unique/stable reagents generated in this study are available from the Lead Contact without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experimental animals
Adult male and female lampreys (Lampetra fluviatilis) caught wild in Sweden were used. Lampreys were kept in an aerated fresh water aquarium at 4°C. Animals were treated according to the Swedish Animal Welfare Act (SFS 1988:534), as approved by the Local Animal Research Committee of Stockholm. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The lamprey reticulospinal synapse is a phasic synapse that operates at low temperatures. The level of spontaneous release is very low (Shupliakov et al., 1997), which makes this model synapse advantageous to study synaptic vesicle clustering mechanisms at rest.
that is more difficult to access in mammalian synapses with high-rate vesicle turnover. Earlier studies have demonstrated that many molecular interactions in this synapse are highly conserved.

**Microbe strains**
All GST-fusion proteins and His$_6$-tagged proteins were expressed in *Escherichia coli* (ER2566; NEB).

**METHOD DETAILS**

**Affinity chromatography and immunoprecipitation**
Detergent extracts were prepared from lamprey brain using established procedures (Evergren et al., 2007; Gad et al., 2000; Pechstein et al., 2010a). Five grams of lamprey brain was minced and homogenized in 10 mL of buffer A (20 mM HEPES (pH 7.4), 50 mM KCl, 2 mM MgCl$_2$, 1% Triton X-100) plus protease inhibitors (Complete protease inhibitor pepsicocktail, Boehringer Mannheim). A postnuclear supernatant of the homogenate was prepared by centrifugation at 2,600 rpm for 10 min at 4°C in an SS-34 rotor (Beckman). Triton X-100 was added to the supernatant to a final concentration of 1%, incubated at 4°C for 30 min, and then centrifuged for 60 min at 60,000 rpm in a Ti-70 rotor (Beckman), and the resulting high speed supernatant was saved as Triton X-100 extract. For pull down and direct binding assays, immobilized GST fusion proteins (20-100 µg) were incubated in a total volume of 500-1000 µL buffer A for 1 h at 4°C with brain extracts or recombinant His$_6$-tagged proteins, respectively in the presence or absence of peptides (100 µM). Following extensive washes samples were eluted with sample buffer, and analyzed by SDS-PAGE and immunoblotting. For immunoprecipitation experiments antibodies were coupled to protein A/G PLUS Agarose (Santa Cruz Biotechnology) and incubated with 4 mg lamprey brain Triton X-100 extract in buffer A plus protease inhibitors (Sigma) in a total volume of 1 mL for 2 h at 4°C. Samples were washed extensively. Complexes were analyzed by SDS-PAGE and immunoblotting.

**Antibodies**
Polyclonal anti-lamprey synapsin I D/IDR-antibodies (for sequence of the antigen, see Figure 1), anti-lamprey intersectin 1 SH3A antibodies, and anti-lamprey amphiphysin SH3 antibodies were raised in rabbits. The higher molecular weight bands recognized by these antibodies was also recognized by the E region antibodies, consistent with the sequence of lamprey synapsin Ia. Antibodies were affinity purified on a Hi-Trap column (GE Healthcare) with the antigen covalently coupled. For data on all other antibodies, see Key Resources Table.

**Plasmids**
The lamprey intersectin 1 SH3 domains were cloned into the pGEX4T-1 vector. All GST-fusion proteins and His$_6$-tagged proteins were expressed in *Escherichia coli* and purified using GST-Bind resin (Novagen) and His-Select Nickel Affinity Gel (Sigma), respectively, according to the manufacturer’s instructions. Cleavage of the GST tag was performed with PreScision Protease following a standard protocol (Sigma-Aldrich). Lamprey HA-synapsin I and truncation and deletion mutants thereof were subcloned into pcHA-MK for mammalian expression. The HA-fusion proteins were expressed in Cos7 cells.

**Peptides**
The following synthetic peptides were used in this study: lamprey synapsin I-derived peptide C$^{514}$QPARQQPAPPROQSSQ$^{630}$ (termed WT peptide) and lamprey synapsin I-derived mutant peptide C$^{514}$QPARQQAAPAQAQSSQ$^{630}$ (termed Mut peptide). An N-terminal cysteine was added for immobilization on solid supports.

**In vitro protein droplet experiments**
Proteins with the indicated concentration were mixed in test tubes 0.1M Tris buffers containing 8g/l NaCl with or without 3% PEG 6000 (Fluka). The lamprey IDR was expressed as a GST fusion protein. A mixture of 10% of Alexa 546 - labeled GST-IDR and 90% unlabeled GST-IDR was used. Protein droplet formation was examined at room temperature on poly-L-lysine (Sigma-Aldrich) - coated microscope slides. Fluorescence images were captured with an LSM 700 confocal microscope (Zeiss) using a 63x (n. a. 1.4) objective and acquired using ZEN software within 3 min after addition of reagents, if not otherwise indicated. Fiji Software was employed to quantify droplet size and area in random field scans. Statistical evaluation of the data was performed with GraphPad Prism 6.0 (GraphStat Software, San Diego, CA, USA).

**In vitro liposome and protein binding assay**
To test if SH3A domain can bind synapsin I attached to liposomes, GST-SH3 domains (human) were immobilized via a rabbit polyclonal GST antibody on Dynabeads M-280 (Invitrogen) pre-coated with secondary sheep anti-rabbit IgGs. Beads were incubated with recombinant rat synapsin Ia and small diameter (60 nm) liposomes of a SV-like lipid composition (Phosphatidylcholine: Phosphatidylethanolamine (PE): Phosphatidylserine: Phosphatidylinositol: Cholesterol = 27: 30: 10: 3: 30; 1% rhodamine-PE) in HEPES-buffered saline (20 mM HEPES, pH 7.4, 200 mM NaCl). Liposome accumulation on Dynabeads was assessed by
fluorescence microscopy using an LSM700 confocal microscope (Zeiss). Data were processed using Volocity software (Improvision). For quantification, the mean fluorescence intensity per bead was calculated from at least 10 fields of view from three independent experiments.

Microinjection of compounds into lamprey reticulospinal axons
Lampreys were anesthetized by immersion in 0,1 g/l tricaine methanesulphonate (MS-222, Sigma) and then decapitated. Trunk segments of the spinal cord were dissected out and transferred to a cooled (7-9°C) bath containing oxygenated lamprey Ringer’s solution. All dissection steps, injections, and physiological recordings were performed at 7-9°C in Ringer’s solution containing: 2 mM HEPES, 109 mM NaCl, 2.1 mM KCl, 2.6 mM CaCl₂, 1.2 mM MgCl₂, 4 mM glucose, 0.5 mM L-glutamine. Indicated antibodies or fusion proteins were coupled with Alexa Fluor 488 and introduced into borosilicate glass injection microelectrodes (concentration approximately 1 mg/ml) and microinjected into the reticulospinal axons with pressure pulses (5-15 psi) of 200 ms duration. Microinjections were monitored with a CCD camera (Roper Scientific, Tucson, AZ or Princeton Instruments, Trenton, NY) connected to an upright fluorescence microscope (Olympus BHX-50) with 10x and 40x water-immersion objectives. The concentration of injected compounds was estimated from the fluorescence intensity in injected axons as described (Andersson et al., 2010) and found to be in the 5 – 50 μM range, with the antibodies in the lower part of the range and GST-SH3 domains in the higher part of the range. Assuming that the synapsin concentration in vesicle clusters is similar to that estimated for rat brain synaptosomes (about 50 μM synapsin for each isoform; (Wilhelm et al., 2014)), we estimate that about 0.1 - 1 active reagents (antibodies/GST-SH3A) per synapsin molecule were sufficient to achieve the described effects, thus being in a comparable stoichiometric ratio. In experiments in which microinjections were performed at rest the axons were microinjected under visual control and identified by fluorescence of the injected compound. An extracellular electrode, placed caudal to the microinjection site in experiments in which axons were stimulated, was used to monitor action potentials. Membrane potentials of the impaled axons were monitored throughout injections.

Spinal cords were fixed at rest, or in control experiments during stimulation, in 3% glutaraldehyde, 4% tannic acid in 0.1 M cacodylate buffer, pH 7.4, for 1 h, followed by incubation in the same fixative without tannic acid overnight. After postfixation in 1% osmium tetroxide for 1 h and dehydration in graded alcohol series, the specimens were embedded in Durcupan ACM (Fluka).

Electron Microscopy
Serial ultrathin sections were cut with a diamond knife (Diatome) on a Leica Ultracut UTC ultramicrotome (Leica), mounted on formvar-coated copper slot grids, counterstained with uranyl acetate and lead citrate, and examined at 80 kV in a Tecnai 12 transmission electron microscope (FEI). Effects of microinjections were analyzed in synapses cut at various distances from microinjection sites (i.e., at different concentrations of injected compounds). At least three regions were examined, within 100 μm, 100-200 μm, 200-300 μm from the injection site. In some cases the regions from 300-500 μm were also investigated. All described effects were reproduced in at least two different spinal cords, in which three to five axons were microinjected.

For quantitative analysis, the number of SVs was determined from middle sections of at least five serially cut synapses. The values for the numbers of SVs were normalized to the length of the active zone, as the number of SVs in the cluster is proportional to the length of the active zone in the reticulospinal synapse (Gustafsson et al., 2002).

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analysis of two groups was evaluated using Student’s t tests. To evaluate the differences between more than two groups one-way analysis of variance (ANOVA) was used with Tukey’s post-test comparing every mean with every other mean. Statistical analysis was performed using GraphPad Prism 6.0 (GraphStat Software, San Diego, CA, USA). Data are presented as mean ± SEM or mean ± SD, as indicated. The significance levels are represented as asterisks (*** - p < 0.001, ** - p < 0.01, * - p < 0.05). All statistical details and the exact values of n can be found in the Legends to figures. No statistical method was used to predetermine sample size. Sample sizes were determined empirically based on our previous experiences or the review of similar experiments in the literature.

DATA AND CODE AVAILABILITY
The datasets supporting the current study in the form of electron micrographs have not been deposited in a public repository because there is no suitable repository for this type of data, but are available from the corresponding author on request. The published article includes all other datasets generated or analyzed during this study.