The reserve pool of synaptic vesicles acts as a buffer for proteins involved in synaptic vesicle recycling

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Presynaptic nerve terminals contain between several hundred vesicles (for example in small CNS synapses) and several tens of thousands (as in neuromuscular junctions). Although it has long been assumed that such high numbers of vesicles are required to sustain neurotransmission during conditions of high demand, we found that activity in vivo requires the recycling of only a few percent of the vesicles. However, the maintenance of large amounts of reserve vesicles in many evolutionarily distinct species suggests that they are relevant for synaptic function. We suggest here that these vesicles constitute buffers for soluble accessory proteins involved in vesicle recycling, preventing their loss into the axon. Supporting this hypothesis, we found that vesicle clusters contain a large variety of proteins needed for vesicle recycling, but without an obvious function within the clusters. Disrupting the clusters by application of black widow spider venom resulted in the diffusion of numerous soluble proteins into the axons. Prolonged stimulation and ionomycin application had a similar effect, suggesting that calcium influx causes the unbinding of soluble proteins from vesicles. Confirming this hypothesis, we found that isolated synaptic vesicles in vitro sequestered soluble proteins from the cytosol in a process that was inhibited by calcium addition. We conclude that the reserve vesicles support neurotransmission indirectly, ensuring that soluble recycling proteins are delivered upon demand during synaptic activity.

buffering | clathrin | synapsin | stimulated emission depletion microscopy | latrotoxin

Synaptic vesicles fuse with the membrane of the nerve terminal during neurotransmitter release and are subsequently retrieved and recycled within a few tens of seconds. Stimulating synaptic preparations in vitro at physiological frequencies results in the repetitive use of only a fraction of the vesicles (up to ≈20%), termed the recycling pool (1, 2). Similarly, we found that living animals only recycled very few vesicles over a few hours (3), which, in contrast to the in vitro situation, constituted only 1–5%. These observations suggest that the bulk of the vesicle population is not involved in recycling at any one time point and therefore may serve another function.

In principle, the excess vesicles could constitute a reservoir for neurotransmitter. However, vesicles contain only a minority of all neurotransmitter molecules in a neuron (4). Additionally, they are essentially "sealed off" (they are inert in terms of neurotransmitter flow), implying that the neurotransmitter contained within nonrecycling vesicles is never used (reviewed in ref. 4), thus rejecting this first hypothesis. A second possibility has been suggested recently in cultured hippocampal neurons, with "reserve" vesicles perhaps participating in spontaneous release (5). However, in vivo these vesicles simply do not release (3). In addition, we have recently demonstrated that the reserve vesicles do not participate in spontaneous release in vitro in preparations such as the neuromuscular junctions (NMJs) of *Drosophila*, frog, and mouse (6), thus also rejecting this second hypothesis.

Another possibility is suggested by the fact that synaptic vesicles require many soluble accessory molecules that cycle between cytosol and membranes, such as synapsins, Rab proteins, or the proteins involved in the formation of the clathrin coat (7, 8). These molecules, shuttling between soluble and membrane-bound forms, may be lost from the synapse by diffusion into the much larger volume of the axon, unless mechanisms exist to retain them. It is therefore conceivable that the surplus vesicles provide a large buffer for retaining these essential proteins and making them available to the recycling vesicles. Under this hypothesis, any protein that has an affinity for vesicular membranes would be retained in the synapse (with the extent of the retention depending on the affinity). Clearly, one could imagine a plethora of other mechanisms to buffer the many soluble proteins needed for vesicle recycling. However, any buffer for a given protein would need to be generated and then controlled in the synapse. The number of pathways for controlling all of the independent buffers would be staggering. In contrast, generating a high abundance of vesicles, fixed near active zones, allows the buffering of all proteins that interact with vesicles (i.e., all proteins involved in recycling).

We therefore analyzed the interaction between vesicles and soluble proteins and found that the vesicles fulfill the basic requirements of a protein buffer, in that (*i*) they bind and enrich a variety of soluble synaptic proteins, and (*ii*) they release these proteins in what seems to be a calcium-dependent process.

Results

Synaptic Vesicle Clusters Bind Soluble Proteins. The first requirement for a protein buffering model would be that the vesicle clusters [formed in 95–99% proportion by nonrecycling vesicles (3)] bind and concentrate soluble vesicle proteins. To investigate this, we fixed and immunostained mouse NMJs (chosen because of the availability of many high-quality antibodies against mouse synaptic proteins) against several soluble targets, and investigated them by stimulated emission depletion (STED) microscopy (9). Unlike conventional microscopy, STED overcomes the normal diffraction limit and allows us to investigate protein distribution within the confined space of the synapse. Unsurprisingly, synapsin, a protein thought to be involved in the clustering of synaptic vesicles (10), was found in the vesicle clusters (Fig. 1*A*). However, many other proteins were also present (Fig. S1). These include proteins involved in exocytosis indirectly (Rab3, rabphilin, CSP) or directly by modulating the SNARE fusion proteins (complexin, NSF), proteins involved in endocytosis (clathrin, dynamin, endophilin, synaptojanin, amphiphysin, AP180, Hsc70), or proteins involved in active zone function (Rim2).

We analyzed the correlation of the protein labels with the synaptic vesicle marker synaptophysin (Fig. 1*B*). The presence of both proteins and vesicles within the confined space of the synapse leads to some level of correlation, even when the two do not interact with each other. To understand the correlation in more

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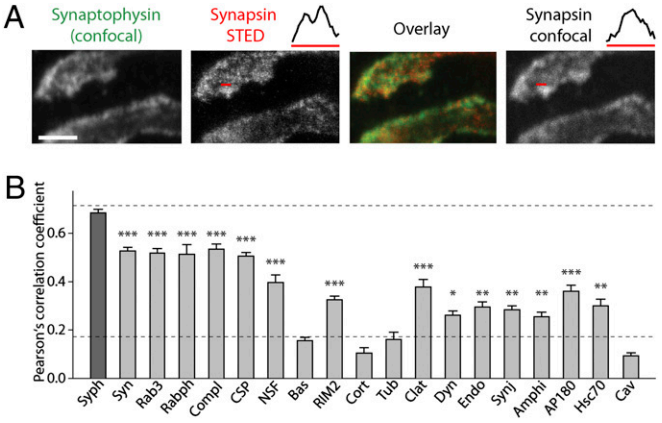

Fig. 1. Synaptic vesicle clusters colocalize with a whole host of soluble accessory proteins. (*A*) Mouse NMJ preparations were immunostained for synaptophysin (a membrane protein of the synaptic vesicle) and for the soluble proteins of interest (depicted here for synapsin). The vesicles were visualized in confocal mode, whereas the proteins of interest were visualized in STED mode (9) (with the STED microscope providing a resolution of ≈70–80 nm in the X-Y plane; Fig. S1). Note that the increased resolution in STED microscopy reveals better the individual protein clusters (line scan). (Scale bar, 2 μm.) (*B*) Pearson's correlation coefficient was determined for synaptophysin and the proteins of interest. Positive control (dark gray): synaptophysin (Syph). Vesicle-associated protein: synapsin (Syn). Proteins involved in exocytosis: Rab3, rabphilin (Rabph), complexin (Compl), CSP, NSF. Active zone proteins: Bassoon (Bas), RIM2. Cytoskeletal elements: cortactin (Cort), tubulin (Tub). Clathrin machinery components: clathrin (Clat), dynamin (Dyn), endophilin (Endo), synaptojanin (Synj), amphiphysin (Amphi), AP180, Hsc70. Protein not associated with vesicles: caveolin (Cav). The graph shows means of 24–162 synaptic areas (±SEM). Dotted lines represent the correlation coefficients expected for a protein distributing randomly in the synaptic volume (0.17) and one fully bound to vesicles (0.71) (more details in Fig. S2). Asterisks indicate significant association of the particular protein with synaptic vesicles (*$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$, *t* test).

detail, we constructed a synapse *in silico*, using a 3D electron microscopy reconstruction from the mouse NMJ. From this reconstruction we generated virtual fluorescence images of both proteins and synaptic vesicles, using the exact parameters (X-Y resolution, Z-resolution, Z-drift) of our experimental setup (Fig. S2). The vesicles were placed in the positions known from the 3D reconstruction, whereas the proteins (which were initially placed randomly in the synaptic volume) were assigned different affinities for the vesicles and were allowed to bind to the vesicles.

As expected, even at no affinity for vesicles, the protein signal overlaps to some extent with that of the vesicles (simply because the two share the same volume), providing a low correlation value (lower dotted line in Fig. 1*B*). This minimal correlation value was similar to that of proteins known not to be enriched on vesicles/vesicle clusters, such as cortactin or tubulin (Fig. 1*B*), confirming the validity of the model. The localization of many other proteins correlated with vesicles at levels significantly higher than those for cortactin or tubulin (Fig. 1*B*). This observation was confirmed by two-color STED microscopy of several proteins (Fig. S3*A*); the synaptic vesicles were again seen to form clusters containing the different proteins we investigated.

Finally, to test whether these proteins indeed bind to the vesicles, we purified synaptic vesicles from rat brain in a procedure relying on size exclusion chromatography, which ensures that at least 95% of the organelles obtained are small synaptic vesicles (11). As reported previously (11), several proteins are enriched on synaptic vesicles (such as CSP, Rab3, synapsin, and rabphilin), whereas most of the clathrin machinery components are also present, albeit in quite low amounts (Fig. S3*B*). Because these vesicles are produced in lengthy purification protocols, lasting more than 30–36 h, one would expect that most proteins that are loosely associated with the vesicles would be lost—which makes it all the more impressive that several proteins still associate to a high extent with the vesicles.

Synaptic Perturbation Results in the Release of Soluble Proteins from Vesicles. The second requirement for the hypothesis that vesicle clusters constitute depots of molecules is that the latter should diffuse out of the synapse when the clusters are disrupted. Treating synapses with black widow spider venom (BWSV) in the absence of calcium triggers synaptic vesicle release while inhibiting their subsequent recycling, causing a marked loss of vesicles (12). We found that it also causes a marked diffusion of soluble proteins into the axons of mouse NMJs (Fig. 2 *A* and *B*), with most of the proteins indicated above being lost from the synapse [Fig. 2*C* and Table S1; we note that the effects of BWSV were reproduced by its main component, latrotoxin (Fig. S4)].

Because the vesicle membrane components are not actually lost from the synapse but fused to the plasma membrane, we expected that many binding partners would still be retained in the synapse. This was the case for some but not all proteins (Fig. 2*C*). Perhaps binding to vesicle membrane of those proteins lost from the synapse is weakened after exocytosis of the vesicle. Reasons for such weakening may reside in lipid composition (13) or reduced membrane curvature, as would be expected, for example, for Bin, amphiphysin, Rvs (BAR)-domain proteins.

Synaptic Vesicles Buffer Soluble Proteins in a Calcium-Dependent Manner. The observations presented above do not explain how efficient buffering could be attained and how the binding/unbinding of many unrelated proteins to the vesicles might be regulated. To investigate this, we first tested whether prolonged in vitro electrical stimulation, which induces substantial vesicle recycling, would also induce the release of proteins from the vesicle clusters. Synapsin, Rab3, and rabphilin were chosen for this investigation, because they provided easily measurable changes in the BWSV/latrotoxin experiments. All were released from the synapses and diffused into the axons upon 5 min of 30-Hz stimulation (Fig. 3 *A* and *B*). They returned into the synapse after the cessation of stimulation [as has already been described for synapsin in cultured neurons (14)], although rabphilin did not recover completely. It should be noted that 5 min of continuous high-frequency stimulation are substantially above the natural activity levels of this preparation.

Does stimulation induce vesicle recycling, which in turn releases proteins from the vesicle cluster, or is the calcium entry caused by stimulation sufficient to release the proteins, independent of vesicle recycling? To test this, we treated the preparations with ionomycin, an ionophore that raises the intracellular levels of calcium but that caused only a mild increase in exocytosis at the NMJ [at a lower concentration than the 10 μM we used here, however (15)]. Ionomycin treatment caused the diffusion of synapsin, Rab3, and rabphilin from the synapses, despite the fact that it did not induce detectable levels of vesicle recycling (Fig. 3 *C* and *D*). Replacing calcium in the extracellular buffer with EGTA (which in the presence of ionomycin lowers intracellular calcium concentrations) caused the return of the released proteins into the synapse. This demonstrates that calcium levels control the release from (and rebinding to) the vesicle clusters for several soluble proteins.

To examine this result using a different assay, we again turned to isolated synaptic vesicles. As indicated above (Fig. S3*B*), vesicles submitted to a lengthy purification procedure still contain many soluble proteins. According to the protein buffering hypothesis, the amount of soluble protein on the vesicles should rise when the vesicles are placed in contact with a source of soluble proteins (such as cytosol). Second, calcium addition should remove soluble proteins from the vesicles. To test this, we incubated synaptic vesicles in vitro with rat brain cytosol, in presence or absence of calcium. We tested the nature of the proteins gained/lost from the vesicle pellet by Western blotting (Fig. 4). Overall, soluble proteins accumulated on the vesicles when these were subjected to cytosol addition (increases of

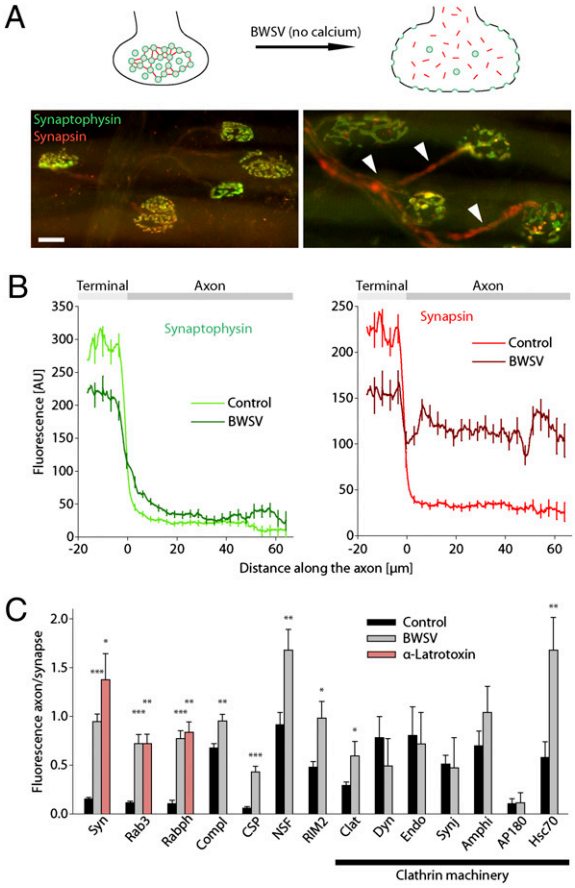

Fig. 2. Soluble proteins diffuse from the vesicles upon synaptic perturbation. (*A*) Vesicle loss induced by BWSV through stimulating fusion in the absence of compensatory endocytosis (12) is followed by the diffusion of synapsin (red) into the axons (arrowheads) in the mouse NMJ. Note that the fused vesicles remain in the synapses (synaptophysin, green). (Scale bar, 20 μm.) (*B*) Quantification of the signal intensity for synaptophysin and synapsin. Graphs show means (±SEM) of at least 99 synapses from three to four independent preparations. Note the increase in synapsin signal in the axon after BWSV incubation. (*C*) Changes in protein distribution after BWSV treatment. Bar graph shows the ratio of fluorescence within the axon compared with the synapse fluorescence, for control (black) and BWSV-treated preparations (gray; 21–112 synapses from two to four independent preparations, ±SEM). Asterisks indicate significant loss of protein into axons (*$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$, *t* test). Note that the clathrin machinery components are not expected to leave the synapse, because they would bind to the fused vesicles, their target. Also note that for synapsin, Rab3, and rabphilin, the results obtained for α-latrotoxin treatment are added for comparison (complete experiment shown in Fig. S4).

≈50–300%; $P < 0.05$, paired *t* test; $n = 14$ proteins) but not in the presence of calcium ($P < 0.05$, paired *t* test; $n = 14$ proteins). Although a full description of the buffering phenotype is beyond the purpose of this work, it is interesting to note that some proteins enriched very highly from the cytosol (5- to 10-fold, as for NSF, Rim, and Hsc70), whereas others were completely removed from the vesicles upon calcium addition (such as amphiphysin or synaptojanin; Fig. S5).

Milder effects could be seen on the complexin levels (Fig. 4*F*), confirming that it does not bind vesicles with high affinity (Fig. S3*B*). This low buffering of complexin is apparently compensated for by the fact that complexin is extremely abundant in the axons under normal conditions (the highest staining in the axons of any of the proteins we analyzed; Fig. S5*F*), an observation that points out the advantage of buffering with respect to the economy in protein use.

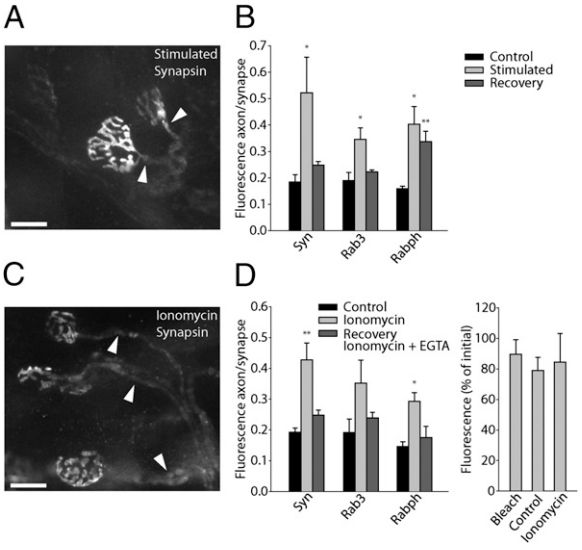

Fig. 3. Soluble proteins diffuse from the vesicles upon calcium influx. (*A*) Electrical stimulation causes the diffusion of soluble proteins into the axon. Mouse NMJ preparations were stimulated for 5 min at 30 Hz; note the abundance of synapsin molecules in the axons (arrowheads). (Scale bar, 20 μm.) (*B*) Quantification of protein distribution after 5 min of stimulation. Asterisks indicate significant loss of protein into axons (*$P < 0.05$, **$P < 0.01$, *t* test). All tested proteins diffuse out of the synapses upon stimulation and return (at least to some extent) after a 20-min period of recovery. Graph shows means ± SEM from three to four experiments. (*C*) The increase in intracellular calcium induced by ionomycin is sufficient to cause the diffusion of soluble proteins into the axon. Preparations were incubated in presence of 10 μM ionomycin in normal mouse buffer for 60 min. Note the abundance of synapsin molecules in the axons (arrowheads). (Scale bar, 20 μm.) (*D*) Quantification of protein distribution after 60 min of ionomycin treatment. Asterisks indicate significant loss of protein into axons (*$P < 0.05$, **$P < 0.01$, *t* test). All tested proteins returned to the synapse after replacing calcium in the extracellular fluid with 5 mM EGTA (in presence of ionomycin, for 60 min), which lowers intracellular calcium. *Right*: Quantification of the effects of ionomycin on synaptic vesicle recycling. Synapses were labeled with FM 1-43 by a 60-s tetanus (30 Hz). The fluorescence remaining after 1 h at room temperature (in presence or absence of ionomycin) was then measured. Note that the decrease in FM 1-43 fluorescence is not significantly different from the photobleaching control (preparations imaged two times, within ≈1–5 min), indicating that ionomycin causes a significant release of soluble proteins from the vesicles, without triggering a measurable increase in vesicle recycling. Graphs show means ± SEM from three to five experiments.

Buffers for Accessory Proteins Are Essential in the Synapse. Although our results do not demonstrate that protein buffering is the only function of the nonreleasing vesicles, they do indicate that these vesicles act precisely as a buffer, according to its definition: they bind and enrich proteins and are able to release them upon activity, which ultimately prevents the diffusion and loss of proteins from the synapses during periods of inactivity (note that the reserve vesicles themselves remain "inactive" throughout, i.e., they do not exocytose). Calcium may trigger the protein unbinding from vesicles through second messenger interactions; however, it is interesting to note that a direct effect of calcium on the clustering of several unrelated plasma membrane proteins (via electrostatic interactions) has been recently reported (16).

Is a buffer for accessory proteins required in the synapse? What types of effects would such a buffer have during activity? Importantly, the presence of clustered vesicles near active zones suggests that they would increase the concentration of molecules available for vesicle recycling. On the other hand these molecules are not available while they are bound to clustered vesicles. To analyze the net outcome of such conflicting features we considered a molecular model, in analogy to Ca^{2+}-buffering by chelators, in which the soluble molecule *X* (such as clathrin)

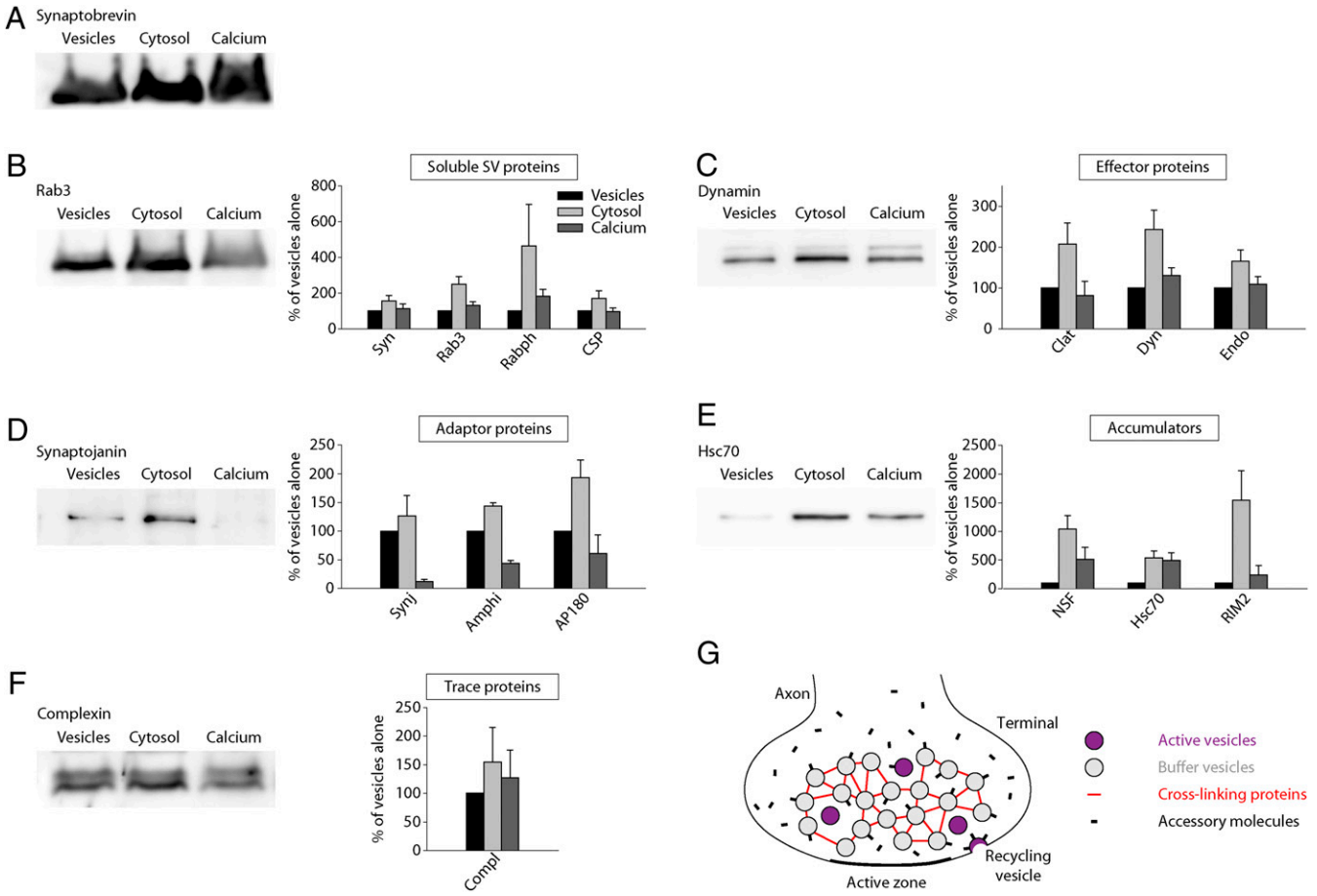

Fig. 4. Biochemical analysis of the binding of soluble proteins to isolated synaptic vesicles. To test whether soluble synaptic proteins unbind from vesicles in presence of calcium, we used isolated synaptic vesicles in vitro. We incubated the vesicles for 30 min at 37 °C in presence of rat brain cytosol, an ATP-regenerating system (+ATP), with or without an addition of Ca^{2+} (1 mM). The three conditions investigated were as follows: vesicles alone ("vesicles"); vesicles + cytosol/ATP ("cytosol"); vesicles + cytosol/ATP + Ca^{2+} ("calcium"). The mixtures were then ultracentrifuged, and the vesicle pellets were analyzed by SDS/PAGE and Western blotting. The amount of vesicles in the pellet is indicated by blotting for synaptobrevin (*A*). Most of the proteins investigated were lost from the pellet upon calcium addition. Several distinct phenotypes could be described: (*B*) Synaptic vesicle proteins (synapsin, Rab3, rabphilin, CSP). Their levels increase upon cytosol addition, with the amount gained being lost upon calcium addition. (*C*) Proteins directly involved in endocytosis (clathrin, dynamin, endophilin). They enrich on vesicles in presence of cytosol and are released again upon calcium addition. (*D*) Endocytosis adaptors or accessory proteins (synaptojanin, amphiphysin, AP180). They are present in lower amounts on the vesicles, are collected from the cytosol, and are almost completely lost from the vesicles upon calcium addition (even below initial levels). (*E*) Proteins collected in high amounts from the cytosol ("accumulators"). They are still present on vesicles after cytosol addition in presence of calcium, to higher levels than on isolated vesicles (although a substantial amount was still lost compared with cytosol addition in absence of calcium). Note that the *y* axis reaches 2,500% for this graph. (*F*) Complexin. It is found only at trace levels on the vesicles (Fig. S3*B*). No substantial effects could be detected. All graphs indicate means ± SEM from three to six measurements, from three to four experiments. Representative immunoblots for all proteins are shown in Fig. S5. All values were corrected for any variations in the amount of vesicle membrane in the pellets (obtained by immunoblotting for the vesicle transmembrane proteins synaptobrevin, synaptophysin, and synaptotagmin; see also Fig. S5). (*G*) Schematic view of the "buffer pool" model. Neurotransmitter release is driven by a few "active" vesicles (purple). The majority of vesicles belong to the "buffer pool" (gray); they are virtually inactive and cross-linked to each other (red). Their function is to buffer molecules involved in vesicle recycling (black).

binds reversibly to the vesicle cluster C, with binding and dissociation rates k_+ and k_-. Additionally, it can bind to recycling vesicles (RV) with rate constant k_d. The source of molecules would be the biosynthetic machinery in the neuronal cell body, whereas the ultimate molecular sink is constituted by the degradation machinery:

$$XC \underset{k_+}{\overset{k_-}{\rightleftharpoons}} C + \begin{array}{c}\text{source}\\ \downarrow k_s\\ X\\ \downarrow k_r\\ \text{sink}\end{array} + RV \overset{k_d}{\rightleftharpoons} XRV$$

The rate of change of X in the absence of recycling ($k_d = 0$) is given by:

$$\frac{d[X]}{dt} = k_s^* - [X](k_r + k_+[C]) + k_-[XC], \qquad [1]$$

where k_s* is the product of k_s with the concentration of the source, which is assumed to be constant.

The rate of change of molecules bound to the cluster (XC) is given by:

$$\frac{d[XC]}{dt} = k_+[X][C] - k_-[XC]. \qquad [2]$$

Because at steady state both $d[X]/dt$ and $d[XC]/dt$ are zero, we can eliminate the terms involving C in Eq. **1** by using Eq. **2**, and we arrive at:

$$0 = k_s^* - k_r[X]. \quad [3]$$

This leads to:

$$[X] = \frac{k_s^*}{k_r} \quad [4]$$

and shows that the steady state concentration of X does not depend on the presence of the buffer, because at steady state the buffer will reach a degree of loading in which the binding to the buffer is balanced by dissociation from the buffer.

In contrast, the speed of changes in the concentration of X in response to external perturbations will be strongly influenced by buffers. For the case that buffer-sites C (e.g., binding sites for clathrin) are not saturated ($[XC] << C^t$, where C^t is the total concentration of C) and that the buffering reaction is fast relative to such perturbations, this effect can be readily described by introducing a buffering ratio κ in analogy to the Ca^{2+}-binding ratio for Ca^{2+} buffers (see refs. 17 and 18):

$$\kappa = \frac{d[XC]}{d[X]} = C^t \frac{K_D}{(K_D + [X])^2}, \quad [5]$$

where K_D is k_-/k_+. Assume a pulse-like perturbation, such as a sudden sequestration of accessory proteins during vesicle fusion and the ensuing clathrin coat formation, would result in a decrement in free clathrin concentration of amplitude A. Then the return to the normal concentration would follow an exponential with time constant τ:

$$\tau = \frac{\kappa}{k_s + k_r}. \quad [6]$$

This is κ-fold longer than the time constant in the absence of the buffer [$\tau_0 = 1/(k_s + k_r)$], whereas the amplitude of the change would be κ-fold smaller than that expected in the absence of the buffer. Thus, the effect of this type of buffering is to mitigate and to slow down changes in the availability of the buffered species upon demand—which is the common sense view of a buffer's role. Contrary to common understanding of buffers, the steady-state free concentration is not set by the buffers (see above). Buffers of this kind exert their main effect if the buffered species C is consumed within a short period to a degree that exceeds its availability as free C within the volume accessible to the reactants.

Strong perturbations are indeed expected to take place in synapses. For example, in the absence of buffers the release of only a few vesicles may result in a deep depletion of free clathrin molecules: in the frog NMJ, one action potential, delivered in vitro in the presence of 4-amino pyridine, released approximately 1% of all vesicles (or 10–20 vesicles for each bouton) (19). This stimulus overwhelmed the endocytic capacity of the terminal: the clathrin immediately available after stimulation (within 1 s) was sufficient only for the formation of approximately 2.5–3.2 coats/μm^2 of nerve terminal face, meaning five to six coated vesicles for an average frog NMJ bouton (19). The remaining vesicular membrane was retrieved by bulk endocytosis and in delayed clathrin-dependent endocytosis (19), suggesting that clathrin is a limiting resource at the synapse and that a buffer may be essential in vivo.

To analyze the requirements for buffering more deeply we will continue to discuss the case of clathrin as an examplar molecule, because it has been investigated in detail in the past (although our arguments are by no means limited to clathrin). Clathrin dynamics have been examined in living cultured neurons by fluorescence imaging (20). Hippocampal neurons were stimulated at 20 Hz for several seconds, and the changes in clathrin-GFP intensity were recorded. Interestingly, after a lag of ≈13 s of stimulation clathrin started to diffuse from adjacent axonal areas into synaptic boutons (20). This recruitment of clathrin then continued until the end of stimulation (≈24 s later). A simple interpretation is that during recycling clathrin triskelia are sequestered by newly forming coated vesicles, which depletes free triskelia, causing the diffusion of axonal clathrin into the synapses. To explain the lag phase, Mueller et al. (20) invoked a "readily retrievable pool" of clathrin coats (already bound to membranes) that would feed endocytosis in the beginning. However, this may be supplemented by clathrin loosely bound to (i.e., buffered by) vesicles in the cluster (see also ref. 21).

Assuming an endocytosis rate of 0.5 to 1 vesicle per second per active zone (22), approximately 7–13 vesicles would be endocytosed before free clathrin starts to decline in the boutons (after the buffer-bound clathrin has also been consumed). At this point approximately 500 triskelia had been consumed in each synaptic bouton [assuming 10 endocytosed vesicles, with each coated vesicle containing ≈50 triskelia (23)]. Given the volume of a nerve terminal of 0.12 μm^3 (24), this would result in a total concentration change of 7 μM. Unless the free concentration of triskelia is as high, we would have to postulate that (apart from the "readily retrievable pool" at the plasma membrane) the majority of the clathrin would have to come from the vesicle buffer. On the other hand, we estimate (*SI Discussion, Section 1*) that a free triskelion concentration of 1 μM is sufficient for the observed speed of coat formation. Thus, the overall demand for the amount of clathrin in a bouton is several-fold higher than the requirement for free clathrin. If all of the required clathrin were in a free, mobile form, not only the bouton but the entire cell would have to be filled with 7 μM clathrin, thus placing a substantial strain on the protein-synthesis machinery of the neuron. Postulating that free clathrin in the above example should not drop substantially, one would request a buffering ratio κ well above 7, probably in the range of 10–20. Buffering ratios can be roughly estimated from the BWSV/latrotoxin experiments (*SI Discussion, Section 3*; values presented in Table S1). We did, indeed, find lower bounds of such estimates above 10 for some of the proteins studied. Generally, these estimates suggest that the presence of a protein buffer in the synapse would lower by several-fold the amount of protein, which needs to be generated by the neurons.

Finally, a spatially clustered buffer (i.e., the vesicle cluster near release sites) may be particularly effective: an evenly distributed buffer would release proteins at average distances from sites of endocytosis too large for diffusion to be sufficiently fast. Again, a rough estimate shows (*SI Discussion, Section 2*) that for the expected diffusion coefficients of clathrin triskelia distances between buffers and sites of consumption not longer than 0.6–1.9 μm are adequate—the length-scale of synaptic boutons.

Discussion

We suggest that the majority of the vesicles in a nerve terminal support neurotransmitter release indirectly, by acting as a molecular buffer (Fig. 4*G*). The majority of neurons have an extended structure and are faced with the problem that in the absence of buffering or other localization mechanisms they would have to maintain a high concentration of soluble proteins throughout the cell volume, even when these are needed only at few, very restricted sites (such as synapses). A certain minimum free concentration of such proteins is required to guarantee fast enough association reactions. Our analysis on diffusion limitation (*SI Discussion, Section 1*) indicates that for clathrin triskelia this might be approximately 1 μM. However, from rates of endocytosis one can postulate that during seconds-long bursts of activity the equivalent of at least 7 μM has to be present in the bouton to build the necessary clathrin coats (*Results*). A buffer that is fully capable to maintain the required free concentration during such activity must have a buffering ratio (bound over free) in the range of 10–100. Two other lines of reasoning point toward similar values for a number of synaptic proteins: first, we show that boutons actually lose large amounts of several synaptic

proteins when being depleted of vesicle clusters. From these measurements we can derive a lower bound to the buffering ratio within boutons (*SI Discussion, Section 3*), which for several proteins is indeed in the range of 10–15 (Table S1), Second, we can postulate that most of the protein synthesized by a neuron should be available within boutons. Given experimental volume ratios (bouton over entire cell), one can readily derive values in the range 100 and more (*SI Discussion, Section 2*). Finally, considering diffusion times between buffers and sites of endocytosis leads to the conclusion that diffusion distances should not be larger than 0.6–1.9 μm, depending on assumptions about the diffusion coefficient (*SI Discussion, Section 2*). This nicely matches with the ultrastructure of synaptic boutons.

The buffering mechanism ensures that endocytosis can happen at sufficient speed, while providing a functional role for the otherwise irrelevant "reserve" vesicles. We concentrated our discussion on clathrin, but synapsin, Rab3, and rabphilin are well-known further examples for proteins that reversibly bind to vesicles (7), with endophilin being recently added to this group (25). The vesicle cluster is the main source within the synapse for all of these molecules, fulfilling all of the basic requirements for a protein buffer—which is possibly its only function, in view of our photooxidation data (3).

The idea that the vesicle clusters contain various synaptic proteins is not new, having already been introduced, albeit in different form (see for example refs. 8 and 26). Previous concepts focused, for example, on clusters releasing molecules when high activity disintegrates them (8); this phenomenon is unlikely to happen in vivo in view of our findings that suggest that clusters stay intact (3). A different hypothesis is that the proteins themselves form a matrix whose function is to retain vesicles in the cluster, to provide a reservoir of vesicles near active zones (8, 26). Our data cannot support this, given that (*i*) no reservoir of vesicles is actually needed, because only a few mobile vesicles function in release, and (*ii*) it is difficult to explain how most of the proteins we investigated could act as vesicle cross-linkers. We therefore suggest that the molecular buffer we envision may be more relevant for the actual function of the vesicle cluster.

As indicated in the Introduction, nonsecretory roles for synaptic vesicles have been considered and proposed before. The vesicles were proposed to provide storage of neurotransmitter, or even of neurotransmitter precursors (as the phoshphatidylcholine contained within vesicular membranes could be used for the acetylcholine released in cholinergic synapses; see for example ref. 27). Although we and others presented arguments against some of these possibilities (Introduction), our suggestion that most vesicles are not directly used in synaptic release is only the latest in a string of such hypotheses. This would have not only an impact on our understanding of synaptic function but may also affect the way we see biological processes in general. Many other cellular entities, such as membrane proteins, are found in clusters for which function is difficult to predict (see ref. 28). We conclude that the paradigm that claims that cellular elements are produced only for their overt function should be viewed with caution. Once a cell has acquired the capability of producing certain organelles or proteins, it may not use them exclusively for their original role but also for other purposes, such as buffering of binding partners. This strategy will allow a more robust fine-tuning of cellular reactions and may increase the resistance of the cells to a wider range of challenges.

Methods

Materials, antibodies, and previously published methods are described in detail in *SI Methods*. Immunostaining/STED microscopy experiments were performed according to standard procedures, as described in *SI Methods* (see also ref. 9). The BWSV treatment of muscles was performed according to the protocols of Henkel and Betz (12), followed by immunostaining and imaging according to standard procedures; see *SI Methods* for more details. Highly purified synaptic vesicles were obtained from rat brain as previously described (11). Either homogenate or synaptic vesicles (7 μg per lane) were investigated by SDS/PAGE/Western blot by conventional methods. Experiments incubating vesicles with cytosolic fractions were performed using crude synaptic vesicles, according to an in vitro protocol previously described (29) and presented in detail in *SI Methods*.

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