

# Synaptic Vesicles Are Constitutively Active Fusion Machines that Function Independently of $\text{Ca}^{2+}$

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## Summary

**Background:** In neurons, release of neurotransmitter occurs through the fusion of synaptic vesicles with the plasma membrane. Many proteins required for this process have been identified, with the SNAREs syntaxin 1, SNAP-25, and synaptobrevin thought to constitute the core fusion machinery. However, there is still a large gap between our understanding of individual protein-protein interactions and the functions of these proteins revealed by perturbations in intact synaptic preparations. To bridge this gap, we have used purified synaptic vesicles, together with artificial membranes containing coreconstituted SNAREs as reaction partners, in fusion assays.

**Results:** By using complementary experimental approaches, we show that synaptic vesicles fuse constitutively, and with high efficiency, with proteoliposomes containing the plasma membrane proteins syntaxin 1 and SNAP-25. Fusion is inhibited by clostridial neurotoxins and involves the formation of SNARE complexes. Despite the presence of endogenous synaptotagmin,  $\text{Ca}^{2+}$  does not enhance fusion, even if phosphatidylinositol 4,5-bisphosphate is present in the liposome membrane. Rather, fusion kinetics are dominated by the availability of free syntaxin 1/SNAP-25 acceptor sites for synaptobrevin.

**Conclusions:** Synaptic vesicles are constitutively active fusion machines, needing only synaptobrevin for activity. Apparently, the final step in fusion does not involve the regulatory activities of other vesicle constituents, although these may be involved in regulating earlier processes. This is particularly relevant for the calcium-dependent regulation of exocytosis, which, in addition to synaptotagmin, requires other factors not present in the vesicle membrane. The *in vitro* system described here provides an ideal starting point for unraveling of the molecular details of such regulatory events.

## Introduction

Synaptic release of neurotransmitters is mediated by the  $\text{Ca}^{2+}$ -dependent exocytosis of synaptic vesicles. In recent years, an increasingly complex network of interacting proteins has been identified that is responsible for the regulation and execution of vesicle docking and fusion. Examples of proteins participating in this network include the SNAREs synaptobrevin, syntaxin 1, and SNAP-25 that catalyze fusion; synaptotagmin, complexin, and Munc-13 that are involved in the  $\text{Ca}^{2+}$ -

dependent control of exocytosis; the SM protein Munc-18 that probably controls SNARE assembly via interaction with other proteins, such as Mints; and an array of protein kinases that phosphorylate proteins such as SNAP-25, providing additional layers of regulation [1].

In contrast to the increasing genetic and physiological evidence linking these proteins to synaptic function, it has been much more difficult to unravel the underlying molecular mechanisms. In fact, there are many cases in which the properties of individual biochemical features are difficult to reconcile with the physiological function of these proteins as studied, for instance, by knockout and knockin approaches. The reason is that there is still a large gap in complexity between perturbing an individual protein in an otherwise intact presynaptic preparation and the study of protein-protein interactions in the test tube with purified components.

Biochemical analysis of supramolecular structures under cell-free conditions can bridge the gap in complexity between these approaches by reconstituting such functional complexes from individual purified components. However, unlike other supramolecular machines, the molecular complexes involved in vesicle docking and fusion are “dirty” nanostructures, in that they involve membranes with highly complex and variable compositions, which recruit proteins only temporarily from the cytoplasm, dissociating once the task is completed [1].

In the synapse, a large body of evidence supports the idea that fusion is catalyzed by the SNARE proteins. According to the zipper hypothesis of SNARE function, the vesicular SNARE synaptobrevin assembles with the SNAREs syntaxin 1/SNAP-25 in the plasma membrane, forming a molecular bridge between the membranes. Assembly involves the SNARE motifs, conserved stretches of 60–70 amino acids that are unstructured in solution but form a bundle containing four parallel  $\alpha$  helices after assembly—the so-called core complex. Nucleation occurs at the N-terminal end of the SNARE motifs and progresses toward the C-terminal membrane anchors, thus tying the membranes together and probably exerting force on the membranes. As a result, repulsion between the membranes is overcome and fusion is induced [1]. Indeed, when appropriate SNAREs are reconstituted in artificial vesicles, they readily fuse in a SNARE-dependent manner [2]. However, other features of exocytosis have not been reproduced in the liposome system. Furthermore, the relevance of such reconstituted systems for biological fusion reactions has been challenged. Liposomes become leaky and fusion-prone at high protein:phospholipid ratios, and they may contain residual detergent that could potentially act as a nonspecific fusion catalyst. Furthermore, liposome fusion can be induced solely by the SNARE transmembrane domains, and vesicles containing synaptobrevin fuse with planar membranes containing syntaxin in the absence of SNAP-25, giving rise to concerns about whether these fusions do indeed represent the molecular intermediates of synaptic exocytosis (reviewed in [3]).

In the present study, we have taken the *in vitro* approach toward reconstituting synaptic exocytosis one step further by investigating the fusion of synaptic vesicles isolated from

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rat brain with liposomes containing the SNAREs syntaxin 1/SNAP-25. By using a native membrane as one of the fusion partners, we hope to avoid potential artifacts caused by leaky or fusion-prone liposomes [3]. Furthermore, purified synaptic vesicles should serve as a benchmark for study of the function of the SNARE synaptobrevin in its native membrane (which, in contrast to liposomes, is loaded with additional proteins that may affect exocytosis). Finally, our recent analysis of synaptic vesicle structure has provided quantitative data for all the major constituents, including synaptobrevin, thus allowing us to match liposomes to their native counterparts [4].

Our results show that synaptic vesicles fuse with such liposomes in a highly efficient and almost quantitative manner. Fusion is mediated by synaptobrevin interacting with syntaxin/SNAP-25 in the membrane, and its kinetics are controlled by the availability of the syntaxin-SNAP-25 acceptor complex. Surprisingly, fusion is not accelerated but rather is slowed by  $Ca^{2+}$ . We conclude that synaptic vesicles are constitutively active fusion machines that are dependent on additional docking and priming factors in order to undergo  $Ca^{2+}$ -dependent exocytosis at the synapse.

## Results

### Synaptic Vesicles Are Constitutively Active Fusion Machines

During initial experiments, we were interested in establishing whether synaptobrevin on synaptic vesicles is free to engage in synaptic core-complexes and, if so, whether core-complex formation could lead to membrane fusion. To avoid potential bias resulting from the method of preparation, we purified synaptic vesicles by two different procedures, involving either size-exclusion chromatography (Method A) or Optiprep floatation gradients (Method B) as the final purification step [4, 5]. SNAREs were reconstituted into liposomes from cholera micellar solutions with a phospholipid composition resembling that of synaptic vesicles and a physiologically relevant SNARE: phospholipid molar ratio of 1:300 [4].

To monitor fusion, we utilized a standard fluorescence dequenching assay, which reports fusion on the basis of lipid mixing [2]. First, we investigated whether synaptic vesicles (purified by Method A) fuse with proteoliposomes containing an N-terminally truncated variant of syntaxin 1A (syntaxinH3) and SNAP-25 in a preformed binary complex (see the [Supplemental Experimental Procedures](#) available online for further details). When synaptic vesicles and liposomes were mixed, a robust dequenching signal was observed (Figure 1A, left hand panel) that developed rapidly and saturated after 10–20 min. To examine whether this dequenching was reporting SNARE-mediated membrane fusion, we used clostridial neurotoxin light chains (which are zinc-dependent proteases) to specifically target the neuronal SNARE proteins [6]. Preincubation with either Botulinum neurotoxin C1 (BoNT/C1) to cleave syntaxin 1A or Tetanus toxin (TeNT) to cleave synaptobrevin resulted in an inhibition of fusion (Figure 1A; see also Figure S1). No inhibition of fusion was observed when toxin mutants were used that had been rendered inactive by an amino acid substitution in the  $Zn^{2+}$ -coordination site. This demonstrates that the fusion reaction requires both intact syntaxin 1 (on liposomes) and synaptobrevin (on synaptic vesicles).

In addition to toxin sensitivity, SNARE-mediated fusion should result in the formation of *cis*-SNARE complexes in the membrane, which are detectable as distinct, heat-sensitive

bands of high molecular mass by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This was indeed the case. Interestingly, vesicular synaptobrevin was almost quantitatively shifted into core complexes when an excess of liposomes was included in the reaction mixture, which is evident by a major loss of the monomeric form (approximately 70% in this case when comparing nonboiled with boiled samples). When the membranes were preincubated with either BoNT/C1 or TeNT, complex formation was largely inhibited (Figure 1A, right-hand panel and data not shown).

Several control experiments were carried out to confirm that fusion between synaptic vesicles and proteoliposomes is dependent on the formation of SNARE complexes. First, we tested whether the fusion kinetics are altered when full-length syntaxin 1 is used instead of syntaxinH3. The N-terminal domain of syntaxin is known to interact with the SNARE motif, downregulating its capability to enter SNARE complexes [7]. However, as shown in Figure 2A, the fusion rate of liposomes containing the full-length protein was only slightly slower than that of liposomes containing the truncated version, suggesting that the N-terminal domain is not rate limiting once SNAP-25 is bound to syntaxin. Lowering of the concentration of vesicles approximately 6-fold reduced both the rate and extent of fusion by about half, with a similar reduction being observed when the liposome concentration was reduced (Figure 1C, left-hand panel). By way of comparison, reliable fusion was also observed with synaptic vesicles prepared according to Method B (Figure 1B and Figure 1C, right hand panel). In addition, fusion of these vesicles was concomitant with the appearance of SDS resistant core complexes, with the majority of synaptobrevin being free to engage in core complexes when an excess of liposomes was included in the reaction mixture (see Figure S4). However, some fusion persisted after incubation with TeNT despite quantitative cleavage of synaptobrevin (Figure 1B).

Further experiments were carried out with synaptic vesicles prepared with Method A, showing that addition of a 1 × molar excess of soluble synaptobrevin, or of soluble syntaxinH3/SNAP-25 (both in 5 × molar excess), effectively inhibited fusion (Figure 2A), as expected for competitive inhibition. Second, no fusion was observed when syntaxin alone was incorporated into liposomes without SNAP-25. Addition of an 8 × molar excess of SNAP-25, either 60 min before or immediately after the start of the reaction, partially restored fusion (Figure 2B). Thus, fusion depends on the formation of SNARE acceptor complexes containing both SNAP-25 and syntaxin 1. No homotypic fusion was observed between syntaxinH3 proteoliposomes or between synaptobrevin-containing proteoliposomes and synaptic vesicles, regardless of whether SNAP-25 was present (Figure 2B). Finally, no nonspecific fusion with protein-free liposomes was detected (see FRET experiments using Oregon Green- and Texas Red-labeled liposomes).

SNARE assembly is thought to overcome the repulsive forces existing between membranes, resulting in fusion. Fusion is thought to proceed via a transient stalk that connects the two membranes. Such stalk-like intermediates possess a negative overall curvature and can be destabilized by amphiphiles possessing an inverted cone shape. Indeed, such lipids have inhibited all fusion reactions examined to date, with lysophosphatidylcholine (LPC) being most commonly used. Addition of increasing amounts of LPC, up to a concentration of 400  $\mu$ M (which is still below its critical micellar concentration [8]), led to a progressive decrease in fusion (Figure 2C).

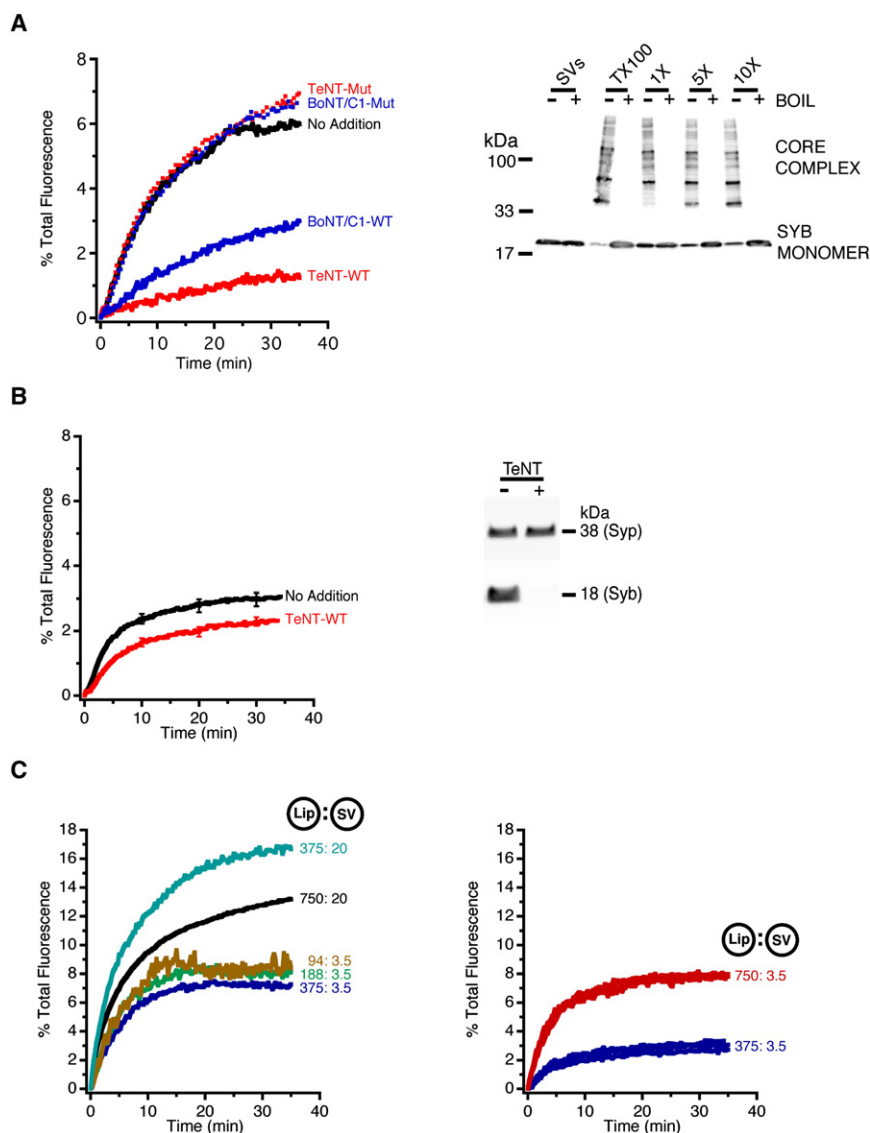


Figure 1. Synaptic Vesicles Fuse Constitutively with Syntaxin 1/SNAP-25 Liposomes, in a SNARE-Dependent Manner, Irrespective of Purification Method

(A) Purified synaptic vesicles (Method A) were added to NBD-Rhodamine-labeled liposomes containing syntaxin 1/SNAP-25. Synaptic vesicles fused constitutively with liposomes, as shown by the robust dequenching signal (No Addition). Preincubation of the membranes (60 min, 37°C) with the light chain of Botulinum neurotoxin C1 (BoNT/C1-WT; cleaving syntaxin 1) or Tetanus toxin (TeNT-WT; cleaving synaptobrevin) largely inhibited fusion. Parallel incubations with light chain mutants, inactivated by a point mutation in the Zn<sup>2+</sup>-coordination site (BoNT/C1-Mut, TeNT-Mut), did not result in any inhibition (left-hand panel). Fusion also resulted in the formation of SDS-resistant, heat-sensitive SNARE core complexes. Increasing the amount of syntaxin/SNAP-25 proteoliposomes (5× and 10× the standard amount) in the reaction mixture increased the amount of synaptobrevin engaged in core complexes. Synaptic vesicles also contain low amounts of endogenous SDS-resistant complexes that were only detectable at longer exposure times (data not shown). An estimate of the maximum number of SDS-resistant complexes that could form was obtained by incubation of proteoliposomes and synaptic vesicles in the presence of 2% (vol:vol) of Triton X-100 (TX100) (right-hand panel).

(B) Robust fusion was also seen between proteoliposomes and synaptic vesicles prepared according to Method B. However, some fusion persisted after incubation with TeNT, despite quantitative cleavage of synaptobrevin (syb). Synaptophysin (syp) is shown as a loading control. Traces are averages of eight experiments ± standard errors of the mean (SEMs).

(C) Fusion was observed for all concentrations of liposomes and synaptic vesicles (Method A left-hand panel; Method B right-hand panel) tested. Figures refer to the final concentration of proteoliposomes (recombinant protein in nM) and amount of synaptic vesicles (total protein in μg) in the reaction mixture.

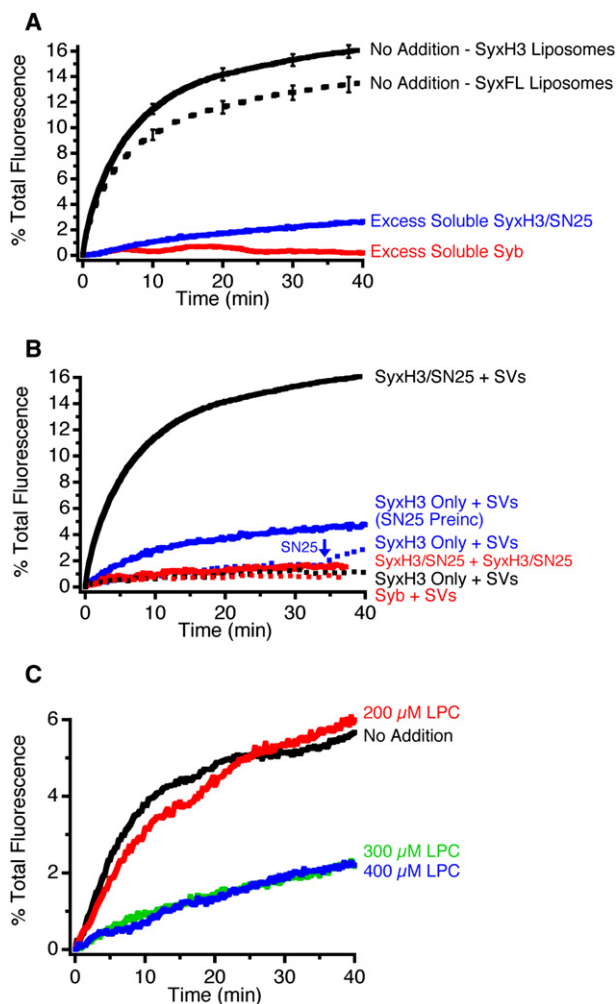
As shown above, the majority of vesicular synaptobrevin is driven into SNARE complexes when an excess of liposomes is used, indicating that all synaptic vesicles isolated by our procedure are essentially fusion competent. To confirm this, we carried out two types of experiments. First, we asked whether fusion results in a detectable size increase. We determined the size distribution of vesicles in a fusion mixture at the end of the reaction and compared it with the size distribution of a fusion reaction that was blocked by preincubation with TeNT. As shown in Figure 3, fusion resulted in a size shift toward larger diameters, although this shift was not as pronounced as that previously observed for synaptobrevin proteoliposomes [9]. However, it needs to be remembered that a single fusion event between vesicles of a similar size only results in a diameter increase of  $\sqrt[3]{2}$ , which is close to the detection limit of electron microscopy. The absence of vesicle clusters in the micrographs suggests that when liposomes and synaptic vesicles meet, fusion is fast with no prolonged docking state preceding fusion (see also Figure S7).

Together, these data show not only that isolated synaptic vesicles effectively fuse with proteoliposomes containing SNAP-25 and syntaxin 1 but also that fusion is SNARE

mediated. Fusion is almost quantitative, in good agreement with our previous observation showing that vesicular synaptobrevin is not restricted in its ability to engage in SNARE complexes with soluble partner SNAREs [10].

### Synaptic Vesicles Undergo Multiple Fusion Events

As shown in Figure 3, fusion leads to the generation of larger vesicles, with diameters ranging between 60 and 80 nm, suggesting that these vesicles may result from multiple fusion events. To investigate this, we used the following fluorescence resonance energy transfer (FRET)-based strategy. First, we fused synaptic vesicles with nonsaturating amounts of syntaxin/SNAP-25 proteoliposomes containing Oregon Green-labeled phospholipids. Next, an excess amount of syntaxin/SNAP-25 proteoliposomes containing Texas Red-labeled phospholipids was added. Because fusion is dependent on synaptobrevin, a FRET signal can only develop if a Texas Red-labeled liposome fuses with a synaptic vesicle that has acquired Oregon Green-labeled lipids in a preceding fusion step (see Figure 4A). Indeed, a FRET signal was observed that increased over the course of the experiment. The signal was small, probably because of the fact that only a small



**Figure 2.** Characterization of Fusion between Purified Synaptic Vesicles and Proteoliposomes Containing Syntaxin 1/SNAP-25, with Fluorescence Dequenching

(A) Fusion of synaptic vesicles with liposomes containing SNAP-25 and either full-length syntaxin 1 (SyxFL) or a truncated syntaxin 1 variant (SyxH3), which lacks the autoinhibitory N-terminal Habc domain, showing that full-length syntaxin is only slightly less active in the lipid dequenching assay. (Traces are averages of at least eight experiments  $\pm$  SEMs). Fusion is inhibited when liposomes and synaptic vesicles are preincubated for 60 min at room temperature with the cytoplasmic fragments of either synaptobrevin (1  $\times$  molar excess) or syntaxinH3/SNAP-25 (5  $\times$  molar excess), respectively.

(B) Fusion of synaptic vesicles to proteoliposomes requires the presence of all three neuronal SNAREs with correct topology. Proteoliposomes produced with a preformed syntaxin/SNAP-25 complex fuse with synaptic vesicles. No fusion was observed with syntaxinH3 proteoliposomes alone. Fusion was partially restored by either incubation of proteoliposomes with an 8  $\times$  molar excess of SNAP-25 relative to syntaxin for 60 min prior to the reaction (SN25 preinc), or by addition of SNAP-25 during the reaction (arrow). No homotypic fusion between syntaxinH3/SNAP-25 proteoliposomes or synaptobrevin proteoliposomes and synaptic vesicles was observed.

(C) Addition of lysophosphatidylcholine (LPC) results in an inhibition of fusion.

amount of Oregon Green-labeled proteoliposomes was used in the initial fusion step. To confirm that the FRET signal was caused by SNARE-mediated fusion, we used protein-free Texas Red liposomes. As shown in Figure 4B, there was no increase in acceptor fluorescence. Donor fluorescence

decreased somewhat, albeit less than under fusion conditions, which is probably attributable to photobleaching. We conclude that under these in vitro conditions, synaptic vesicles are capable of undergoing multiple rounds of fusion. Synaptobrevin molecules that were not involved in the first fusion reaction retain their ability to undergo subsequent rounds of fusion (see Figure 1A, right-hand panel and Discussion).

### Synaptic Vesicle Fusion Is Independent of $Ca^{2+}$

The data described so far show that synaptic vesicles effectively fuse with liposomes containing syntaxin/SNAP-25. At the synapse, exocytosis is triggered by a rise in intracellular  $Ca^{2+}$  during stimulation. Synaptic vesicles contain at least ten copies of the  $Ca^{2+}$ -sensor synaptotagmin 1, so we wondered whether fusion is accelerated by the addition of  $Ca^{2+}$  ions. Surprisingly, no increase in the fusion rate was observable (Figure 5A). Rather, a minor inhibition was seen, even at concentrations known to stimulate exocytosis in neurons. A similar inhibition was also observed when the  $Ca^{2+}$  concentration was raised to (nonphysiological) 1000  $\mu$ M [11]. This inhibition was partially prevented when phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) was included in the membrane of the liposomes (Figure 5B), although the effects of  $PIP_2$  inclusion were rather minor, even when it was systematically varied over a large range (see Figure S6). Finally, addition of the cytoplasmic domain of synaptotagmin to the fusion reaction produced only minor effects on the fusion rate, regardless of whether  $Ca^{2+}$  was present or not (data not shown). The inability of  $Ca^{2+}$  to stimulate fusion, despite the presence of endogenous synaptotagmin, shows that regulated exocytosis requires additional, hitherto unknown, control proteins (see Discussion).

### The Rate of Synaptic Vesicle Fusion Is Determined by the Availability of Synaptobrevin Acceptor Sites

In the final set of experiments, we investigated whether the rate of vesicle-liposome fusion is dependent on the status of the acceptor SNAREs in the liposome membrane. When SNAP-25 and syntaxin 1 are mixed in vitro, an unstable 1:1 complex is formed that readily recruits a second syntaxin molecule, with the equilibrium being strongly on the side of the 2:1 complex. Because the rate of SNARE assembly and SNARE-mediated liposome fusion depends on the concentration of free binding sites for synaptobrevin, the reaction can only proceed if the second syntaxin molecule (that blocks the synaptobrevin binding site) dissociates from the binary complex [12].

Two approaches were used to increase the concentration of synaptobrevin acceptor sites on the syntaxin/SNAP-25 proteoliposomes. First, complexes were preformed with increasing concentrations of SNAP-25, with the goal of increasing the concentration of 1:1 complexes by mass action. As shown in Figure 6A, a moderate acceleration was observed when the SNAP-25 concentration was doubled, with no further increase at higher concentrations. Second, we took advantage of the recent observation that a free N-terminal acceptor site for synaptobrevin can be stabilized if a SNARE complex is formed containing N-terminally truncated synaptobrevin (referred to as  $\Delta$ N complex), which greatly enhances liposome-liposome fusion [13]. Therefore, we asked whether the fusion of synaptic vesicles with liposomes containing  $\Delta$ N complexes is also accelerated. As shown in Figure 6B, this is indeed the case, with the degree of acceleration comparable to that previously observed when synaptobrevin-containing liposomes were

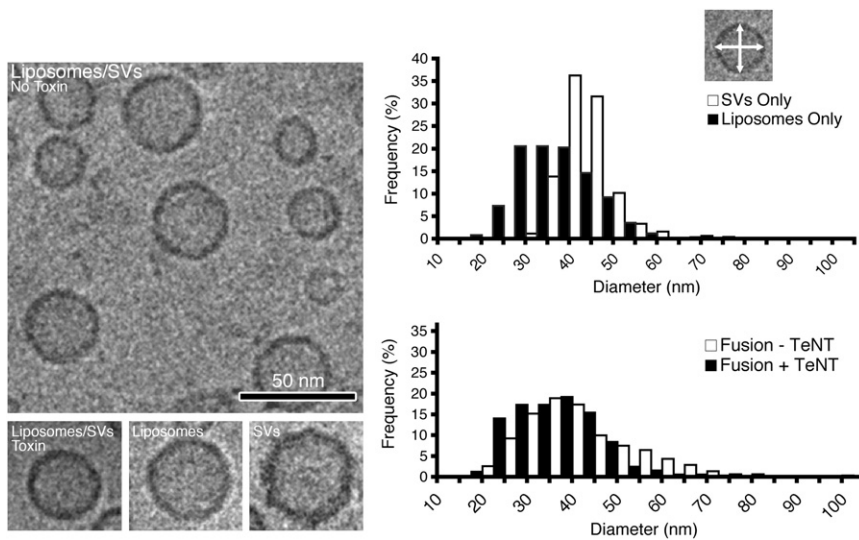


Figure 3. Fusion between Synaptic Vesicles and Liposomes Results in a Size Increase

Cryoelectron micrographs of proteoliposomes, synaptic vesicles, and particles resulting from a fusion reaction (left). Quantification of particle sizes, at the end of a 30 min fusion reaction is shown (right). The longest and the shortest diameters (outer rim) of each particle were measured, and the average diameter was calculated (arrows on inset). The size distributions of the individual proteoliposome and synaptic vesicle populations are shown (top histogram). Fusion resulted in a shift to larger diameters (bottom histogram). As a control, a parallel reaction was made with Tetanus toxin-treated synaptic vesicles, in which no size shift was seen. Interestingly, few particle clusters were seen, indicating that fusion is fast and complete. The experiment was repeated twice and the numbers of particles counted for each condition were pooled. Particle numbers (and the associated standard deviations) are the following: liposomes 688 (8.81); synaptic vesicles 410 (6.37); liposomes and synaptic vesicles -TeNT 647 (14.02); liposome and synaptic vesicles +TeNT 741 (10.36).

used. As expected, fusion was associated with the displacement of the N-terminally truncated peptide (see Figure S7). We conclude that isolated synaptic vesicles are highly efficient fusion machines whose fusion kinetics are determined by the availability of SNARE acceptor sites for synaptobrevin.

## Discussion

We have demonstrated that isolated synaptic vesicles effectively fuse with liposomes containing the cognate neuronal SNAREs syntaxin 1/SNAP-25. Fusion is dependent on the formation of SNARE complexes but is not accelerated by calcium or  $PIP_2$ , despite the presence of native and intact synaptotagmin in the vesicle membrane. Fusion is highly efficient, with most vesicles capable of fusing at least once, and fusion kinetics are predominately governed by the availability of an appropriately configured acceptor complex of syntaxin 1/SNAP-25.

Our data lend strong support to the notion that SNAREs function as the minimal fusion machinery. Our results support the view that synaptic vesicle exocytosis requires all three neuronal SNAREs in correct topology, with at least two SNAREs containing transmembrane domains. Our data also suggest that vesicular synaptobrevin does not need to be activated in order to engage in fusion-competent SNARE complexes with syntaxin 1 and SNAP-25. This conclusion disagrees with several previous studies reporting that synaptobrevin—either in native synaptic vesicles or after reconstitution of the recombinant protein into liposomes—is inactive with respect to SNARE interactions [5, 14]. Upon reinvestigation of this issue, we have been unable to reproduce these findings. Rather, we observed that synaptobrevin anchored in native or artificial membranes can be quantitatively driven into SNARE complexes by addition of exogenous SNAP-25 and syntaxin 1 [2, 10]. Our present findings are fully consistent with these data and confirm that synaptobrevin is constitutively active on the vesicle membrane. Furthermore, several lines of evidence including lipid mixing (coupled to the degree of SNARE complex formation) and size increase measured by electron microscopy conclusively document that a large majority, if not all, of purified vesicles are competent for

SNARE-mediated fusion. The reasons for these discrepancies are unclear at present, although they are possibly caused by differences in the purification of the SNARE proteins, which in some studies involved harsh denaturing steps, or by the use of a high protein:lipid ratio (1:10) during proteoliposome preparation, which may result, among other things, in steric hindrance of the proteins [5].

Our data also show that only a fraction of the synaptobrevin molecules on each vesicle are engaged in SNARE complexes during each fusion event. This is not surprising because each vesicle contains 70 copies of synaptobrevin [4], many more than are thought needed for fusion [15]. This observation explains why vesicles can undergo multiple rounds of fusion and is in agreement with previous findings on a N-ethylmaleimide-sensitive factor (NSF) mutant in *Drosophila*, showing that synaptic vesicles can still undergo several rounds of exo-endocytotic cycling when the disassembly of SNAREs is inhibited [16].

One of our most surprising findings is that neither calcium nor  $PIP_2$  in the liposome membrane affects the fusion rate to a major degree, despite the fact that synaptic vesicles contain endogenous synaptotagmins [1]. In this respect, our findings disagree with the previous report by Hu and colleagues, in which fusion between vesicles and liposomes only took place when calcium was present [5]. In our hands, we could consistently and reliably see fusion, irrespective of the method used to prepare vesicles, and although we did see an enhancement of fusion in the presence of  $Ca^{2+}$  when using vesicles prepared with Method B, we found this enhancement is unrelated to fusion mediated by synaptic SNAREs (see Figure S5). Furthermore, our data are consistent with previous reports in which no calcium-dependent acceleration was observed in SNARE-dependent liposome fusion when full-length synaptotagmin was coreconstituted with synaptobrevin [17, 18]. In fact, in our previous study, we observed a  $Ca^{2+}$ -dependent inhibition of proteoliposome fusion in the presence of synaptotagmin, which is reminiscent of the inhibition reported here [18]. More detailed analysis revealed that this inhibition is due to the  $Ca^{2+}$ -dependent binding of synaptotagmin to its own membrane when acidic phospholipids are present (as they are in synaptic vesicles) [4]. This binding apparently

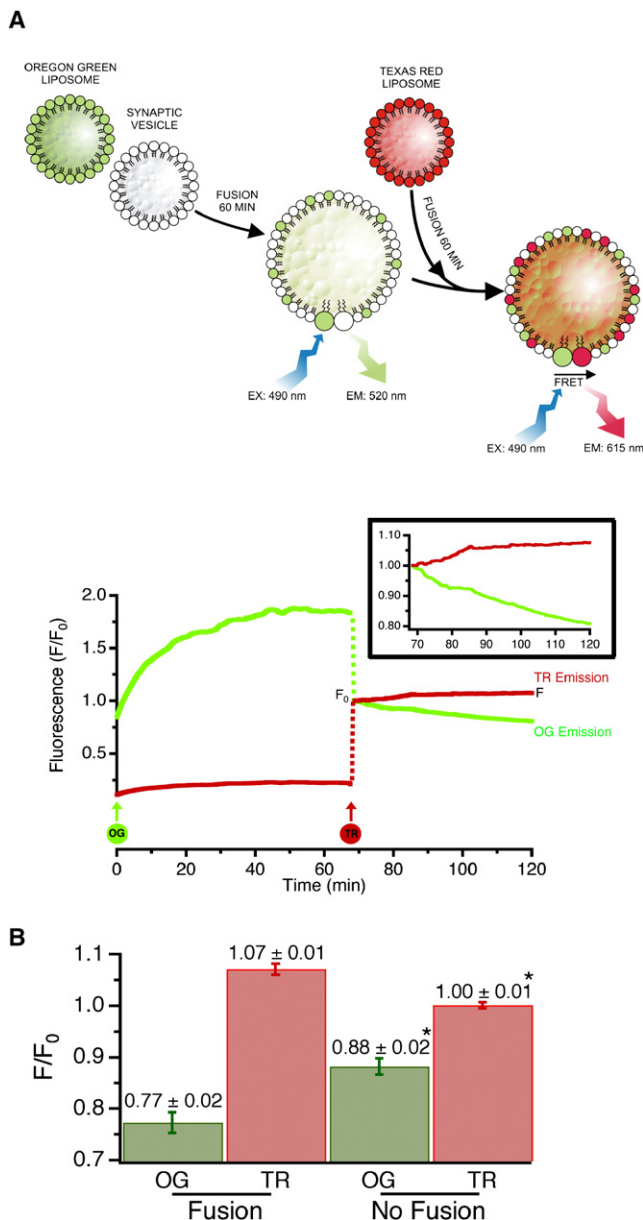


Figure 4. Synaptic Vesicles Undergo Multiple Rounds of Fusion

(A) Synaptic vesicles were first fused with syntaxin3/SNAP-25 proteoliposomes containing Oregon Green-labeled lipid and then with proteoliposomes containing Texas Red-labeled lipid. Because fusion is strictly dependent on one of the fusion partners containing synaptobrevin, FRET only develops if a Texas Red liposome fuses with a synaptic vesicle that has acquired Oregon Green-labeled lipids in a preceding fusion step. FRET was monitored fluorometrically with excitation at 490 nm and emission at both 520 nm (Oregon Green donor, green trace on graph) and 615 nm (Texas Red acceptor, red trace on graph). Syntaxin3/SNAP-25 proteoliposomes, labeled with Oregon Green or Texas Red, were added to synaptic vesicles at the indicated time points (circles OG and TR). The increase in OG fluorescence at the beginning of the experiment is due to dequenching of the dye on fusion. Both OG and TR fluorescence signals were normalized to an arbitrary value of 1 after the TR liposomes were added, to account for the dilution effect (dashed lines;  $F_0$ ). The inlay shows a portion of the graph expanded after addition of TR liposomes to show the decrease in OG (donor) and increase in TR (acceptor) signals typical of FRET.

(B) Donor and acceptor emission signals at the end of the reaction (F) normalized to the point at which the TR proteoliposomes were added ( $F_0$ ). Addition of TR proteoliposomes to the reaction mixture caused a decrease in the OG signal and a concomitant increase in the TR signal (Fusion;  $n = 12$ ,

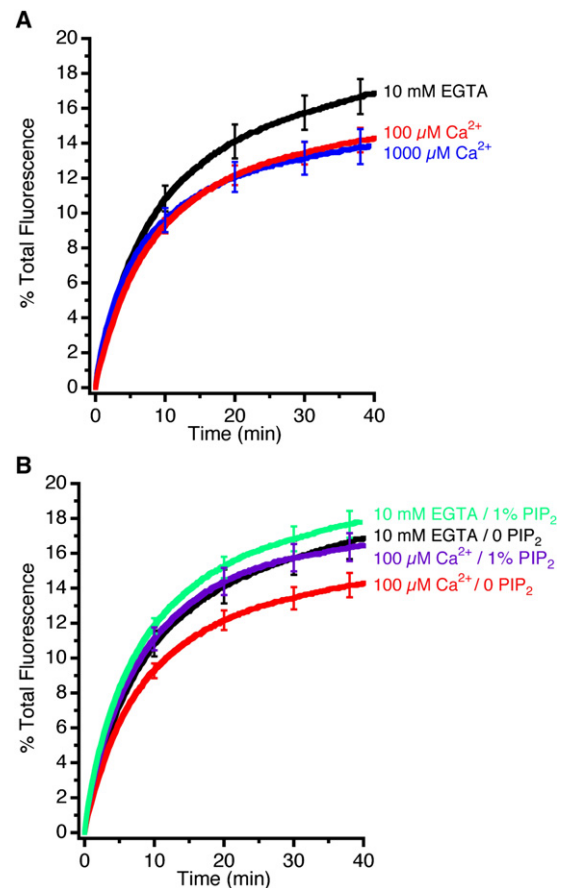


Figure 5.  $Ca^{2+}$  Does Not Accelerate Fusion between Synaptic Vesicles and SNARE-Containing Liposomes

(A) Fusion of synaptic vesicles with syntaxin/SNAP-25 proteoliposomes, as measured by lipid dequenching, is not enhanced by calcium. In fact, a very reproducible inhibition was observable with both 100  $\mu$ M and 1000  $\mu$ M  $Ca^{2+}$ .  $n \geq 6$  experiments  $\pm$  SEMs.

(B) Inclusion of a synaptotagmin effector lipid (phosphatidylinositol 4,5-bisphosphate [ $PIP_2$ ]) in the liposomal membrane did not produce a significant increase in the rate of fusion, as measured by lipid dequenching. However, the inhibition of fusion seen upon the addition of  $Ca^{2+}$  was partially prevented by the incorporation of 1%  $PIP_2$  in the proteoliposome membrane.  $n \geq 9$  experiments  $\pm$  SEMs.

inactivates synaptotagmin, preventing it from interacting *in trans* with syntaxin/SNAP-25 and/or acidic phospholipids in the target membrane. In our hands, the effect of  $PIP_2$  in partially preventing this  $Ca^{2+}$ -dependent inhibition suggests that increasing the amount of this phospholipid in the acceptor liposome is sufficient to re-establish some *trans* interaction between liposome and synaptic vesicle, that is mediated by synaptotagmin [19].

Several conclusions can be drawn concerning the *in vitro* reconstitution of exocytotic membrane fusion with isolated native membranes or artificial vesicles. First, isolated and purified synaptic vesicles exhibit properties virtually identical to proteoliposomes containing comparable amounts of synaptobrevin and synaptotagmin, when liposomes containing syntaxin/SNAP-25 are used as fusion partners. In both cases, fusion proceeds with comparable kinetics (being primarily

average  $\pm$  SEMs), when compared to protein free liposomes (No Fusion;  $n = 11$ , average  $\pm$  SEMs) ( $*p < 0.001$ ).

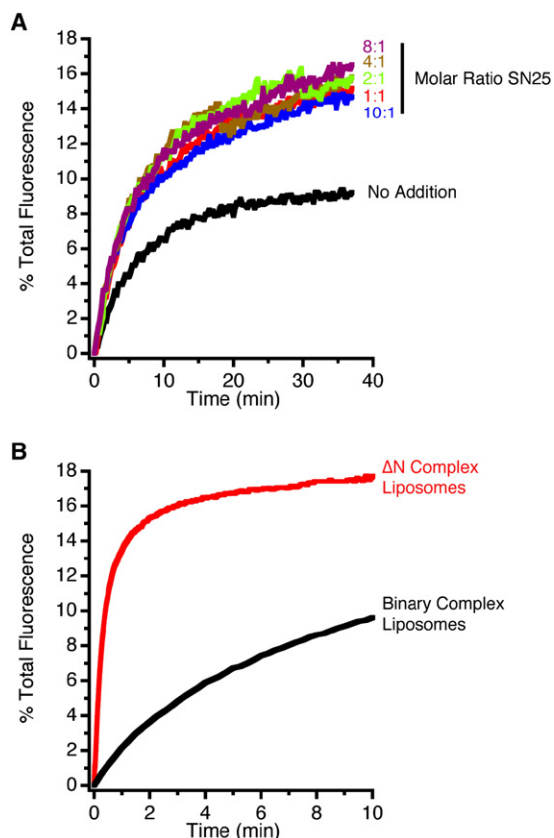


Figure 6. The Rate of Synaptic Vesicle Fusion Is Determined by the Availability of Free Synaptobrevin Acceptor Sites

(A) Increasing of the concentration of free SNAP-25 accelerates vesicle-liposome fusion by shifting the equilibrium by mass action from the energetically favored 2:1 syntaxin/SNAP-25 complex to the unstable 1:1 syntaxin/SNAP-25 complex, which serves as an acceptor for synaptobrevin.

(B) Fusion of liposomes with synaptic vesicles, as measured by lipid dequenching, is greatly accelerated when a syntaxin/SNAP-25 acceptor complex is used that contains N-terminally truncated synaptobrevin (amino acids 49–96, ΔN complex), which leaves a stabilized, freely accessible N-terminal binding site for synaptobrevin. Note that the time scale in (B) is expanded in comparison to that in (A).

dependent on the availability of SNARE acceptor sites), is close to being quantitative (with multiple rounds of fusion occurring), and is moderately inhibited in the presence of calcium. These striking similarities are surprising, when one considers that in contrast to artificially prepared proteoliposomes, synaptic vesicles are loaded with other proteins and have a highly complex, probably asymmetric, lipid composition. Apparently, the massive release of energy during SNARE complex formation is capable of driving fusion under a wide variety of conditions, being able to plough through densely packed cohorts of membrane proteins, as well as accommodating a large variety of membrane lipids. The data also indicate that the in vitro fusion of liposomes is not compromised by an artificially high fusogenicity, as has recently been suggested [3].

Second, the data show that the regulatory steps involved in controlling neuronal exocytosis have not yet been adequately reconstructed in artificial fusion systems. Most importantly, the fast triggering by  $Ca^{2+}$  of exocytotic membrane fusion, which has been unequivocally shown to be mediated by synaptotagmins 1 and 2 [1], has not been reproduced in such

fusion assays. Apparently,  $Ca^{2+}$  triggering requires the synaptic vesicle to be docked and primed at the release site, a molecular configuration that is not yet understood (for a more detailed description, see Stein et al. [18]).

Several additional proteins, such as Munc-18 and complexin, are also known to be crucial for neuronal exocytosis, even though their precise role is not understood. Our present understanding is that these proteins are recruited from the cytoplasm to the site of fusion, where they are known to bind to free and/or assembled SNAREs. Munc-18 has been suggested to regulate the availability of SNAREs in the target membrane, whereas complexin, which binds to assembled SNAREs, has been proposed to clamp the SNARE complex in a prefusion state ready for  $Ca^{2+}$ -dependent exocytosis, suggesting a cooperative action with synaptotagmin [1]. These interactions clearly add yet another layer of regulation. Obviously, more work is needed in order to reconstruct these and other, as yet unknown, regulatory steps in the test tube. However, it seems that such regulation is mediated primarily by factors that are either located at the plasma membrane or that are recruited from the cytoplasm, whereas isolated synaptic vesicles lack endogenous control proteins and are constitutively active for fusion.

#### Experimental Procedures

Experimental procedures were all based on published protocols (see references). Brief outlines are given below, with fuller descriptions being found in the Supplemental Data.

#### Recombinant Protein Expression and Purification

SNAREs were cloned with rat (*Rattus norvegicus*) sequences as templates. SNARE proteins were expressed in *E. coli* and purified by  $Ni^{2+}$ -NTA affinity chromatography, and ion exchange chromatography on an Äkta system (GE Healthcare) [12]. Purification of proteins containing a transmembrane domain was done in the presence of 15 mM CHAPS [18]. Tetanus toxin and Botulinum toxin (including respective mutants) were purified with a similar strategy. The concentrations of all recombinant proteins were determined by absorption at 280 nm.

#### Preparation of Artificial and Native Membranes

##### Proteoliposome Preparation

Lipids were mixed in chloroform to give (in molar ratios) phosphatidylcholine (5), phosphatidylethanolamine (2), phosphatidylserine (1), phosphatidylinositol (1), and cholesterol (1). In liposomes containing PIP<sub>2</sub>, the level of phosphatidylinositol (PI) was reduced accordingly. For use in fluorescence-based assays, unlabeled phosphatidylethanolamine (PE) was substituted in part by N-(7-nitro-2,1,3-benzoxadiazol-4-yl) PE (NBD-PE) and Rhodamine-PE (lipid dequenching), and Oregon Green-PE or Texas Red-PE (FRET assays). After drying, lipids were resuspended in HB100 buffer (100 mM KCl, 1 mM DTT, 25 mM HEPES [pH 7.4, KOH]) with 5% sodium cholate at a total lipid concentration of 13.5 mM. SNARE proteins were added (at a protein:lipid ratio of 1:300), and detergent was removed by gel filtration chromatography, either on a SMART system with a PC 3.2/10 Fast Desalting column (GE Healthcare) or a Bio-Rad EconoColumn filled with Sephadex G-50 superfine. Columns were equilibrated in HB100 [9, 18].

##### Synaptic Vesicle Purification

Synaptic vesicles were isolated from rat brain according to two different procedures. However, both procedures utilize standard differential centrifugation to initially isolate synaptosomes, from which synaptic vesicles are released by hypo-osmotic shock. In Method A, synaptic vesicles are then enriched by differential centrifugation, prior to purification on continuous sucrose density gradients and a final step of size-exclusion chromatography with controlled pore glass beads [4]. In Method B, by way of contrast, large membrane fragments produced during lysis are subsequently removed by differential centrifugation, before the separation of synaptic vesicles from any remaining cytosolic components by floatation on Optiprep gradients [5].

A modified Lowry procedure was used to determine concentrations of membrane proteins.

#### Fluorescence Assays

Liposome fusion measured by lipid dequenching was carried out by incubating NBD-Rhodamine-labeled syntaxin/SNAP-25 liposomes with synaptic vesicles at 37°C. Fusion was reported by the increase in NBD fluorescence (measured at 460 nm excitation and 538 nm emission in a Fluoromax-2 spectrometer) that occurs on lipid mixing. At the end of the reaction, Triton X-100 was added to completely solubilize the membranes and maximally dequench NBD fluorescence. For normalization of the experiments, the lowest NBD fluorescence signal was set to 0% and the maximal signal reached after detergent addition was set to 100% [2, 9].

When FRET was used to study the fusion reaction, Oregon Green-labeled syntaxin/SNAP-25 liposomes (donor) were incubated with synaptic vesicles for 60 min, at which time fusion, as measured by Oregon Green dequenching, had proceeded to completion. At this point, a saturating amount of Texas Red-labeled syntaxin/SNAP-25 liposomes (acceptor) was added and the reaction left for a further 60 min. The donor signal (excitation 490 nm; emission 520 nm) and the acceptor-FRET signal (excitation 490 nm; emission 615 nm) were measured in a Fluoromax-2 at 37°C. The final donor and FRET signals (F) were then expressed relative to the point at which the Texas Red liposomes were first added (F<sub>0</sub>) [20].

All data analysis was performed with Igor Pro (Wavemetrics).

#### Electron Microscopy

Reaction mixtures were applied to carbon-coated grids, plunge frozen in liquid ethane and transferred to a Philips CM120 Biofilter electron microscope with a Gatan cryostage. Images were recorded on a Gatan slow-scan CCD at a magnification of 31,000× with Digital Micrograph software, at a constant defocus. Micrographs were then digitised with a flat-bed scanner at a resolution of 1200 dpi, and the shortest and longest diameters of each particle were measured [9].

#### SDS-PAGE and Immunoblotting

SDS-PAGE was carried out with a glycine- or tricine-based buffer system to ensure maximum resolution in the molecular weight range of interest [9, 21]. Immunoblotting was carried out using standard semidry electrophoretic techniques [22]. Detection was by enhanced chemiluminescence with a LAS reader (Fuji). Primary antibodies were from Synaptic Systems or Abcam. Peroxidase-labeled secondary antibodies were from Bio-Rad.

#### Supplemental Data

Additional Experimental Procedures and seven figures are available at <http://www.current-biology.com/cgi/content/full/18/10/715/DC1/>.

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