In vitro system capable of differentiating fast Ca\textsuperscript{2+}-triggered content mixing from lipid exchange for mechanistic studies of neurotransmitter release

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Understanding the molecular principles of synaptic vesicle fusion is a long-sought goal. It requires the development of a synthetic system that allows manipulations and observations not possible in vivo. Here, we report an in vitro system with reconstituted synaptic proteins that meets the long-sought goal to produce fast content release in the millisecond time regime upon Ca\textsuperscript{2+} triggering. Our system simultaneously monitors both content and lipid exchange, and it starts from stable interacting pairs of donor and acceptor vesicles, mimicking the readily releasable pool of synaptic vesicles prior to an action potential. It differentiates between single-vesicle interaction, hemifusion, and complete fusion, the latter mimicking quantized neurotransmitter release upon exocytosis of synaptic vesicles. Prior to Ca\textsuperscript{2+} injection, the system is in a state in which spontaneous fusion events between donor and acceptor vesicles are rare. Upon Ca\textsuperscript{2+} injection, a rapid burst of complete fusion events emerges, followed by a biphasic decay. The present study focuses on neuronal SNAREs, the Ca\textsuperscript{2+} sensor synaptotagmin 1, and the modulator complexin. However, other synaptic proteins could be added and their function examined. Ca\textsuperscript{2+} triggering is cooperative, requiring the presence of synaptotagmin, whereas SNAREs alone do not produce a fast fusion burst. Manipulations of the system mimic effects observed in vivo. These results also show that neuronal SNAREs alone do not efficiently produce complete fusion, that the combination of SNAREs with synaptotagmin lowers the activation barriers to full fusion, and that complexin enhances this kinetic control.

N euronal communication is made possible by the release of neurotransmitters, which in turn depends on the fusion of neurotransmitter-containing vesicles with the active zone in axonal terminals. Synaptic vesicle fusion is triggered by an influx of Ca\textsuperscript{2+} ions into the neuron upon depolarization. Neurotransmitter release is quantized (1); that is, it involves a few to tens of individual synaptic fusion events. The process of individual synaptic vesicle fusion is in turn controlled by a set of relatively few proteins, such as the SNARE proteins (2–5), the Ca\textsuperscript{2+} sensor for fast synchronous release synaptotagmin 1 (6–8), and the modulator complexin (9–11). Thus, neurotransmitter release is a macroscopic biological phenomenon that is ultimately controlled by a few individual molecules. The understanding of the underlying molecular mechanisms thus requires methods that are inherently capable of observing single vesicles and single molecules (12, 13).

Ideally, observations of single vesicles and single molecules would be performed in live neurons. Although progress for such studies has been made (14), they currently only provide limited information because the necessary genetic manipulations or labeling techniques may not provide the spatial and time resolution required for studying the dynamics of neurotransmitter release. Thus, there is an urgent need to develop minimal synthetic systems that, at least qualitatively, have the neurotransmitter release characteristics observed in neurons and that allow manipulations and observations not possible in vivo.

Previous attempts of in vitro reconstitutions of neurotransmitter release had fundamental limitations. Most of these previous systems did not directly probe the release of synaptic vesicle content, but rather the exchange of lipids between membranes. These experiments were based on the misconception that membrane lipid exchange (also referred to as “lipid mixing”) is indicative of content release (also referred to as “content mixing”). Lipid mixing (which causes partial fusion of membranes) is necessary, but not sufficient for pore formation between membranes and subsequent content release. For example, lipid mixing can occur without content mixing (15, 16), a phenomenon that has been observed in viral fusion where lipid mixing occurs several seconds before content mixing (17, 18), and vacuolar fusion (19). Furthermore, even inner leaflet mixing can occur without content mixing (16). There are other limitations of these earlier in vitro studies: Often only a subset of some of the key constituents were used, and all previous assays utilized a constant Ca\textsuperscript{2+} concentration, rather than a stepwise increase. It addition, synaptic vesicle fusion processes are inherently heterogeneous, so it is important to use single-particle and single-molecule techniques (12, 13) because observation of the average of many such vesicles may obscure an accurate view of the underlying processes.

Here, we describe a synthetic single-vesicle system with reconstituted synaptic proteins that overcomes the limitations of the previous studies. Our system addresses the long-standing challenge to produce a millisecond content release burst upon Ca\textsuperscript{2+} triggering, as monitored by a simultaneous content and lipid-mixing assay with single vesicles. It starts from stable interacting pairs of donor and acceptor vesicles, mimicking the so-called readily releasable pool of synaptic vesicles prior to Ca\textsuperscript{2+} injection (20). The system differentiates between single-vesicle interaction,
hemifusion (or membrane lipid exchange), and complete fusion (i.e., pore formation), the latter mimicking quantized neurotransmitter release upon exocytosis of synaptic vesicles. Because single vesicles are observed and the time course of individual fusion events are monitored, the observations are analogous to quantized Ca\(^{2+}\)-triggered neurotransmitter release of one or more synaptic vesicles. We limited this study to neuronal SNAREs [specifically, synaptobrevin-2, syntaxin-1A, and synapsosomal-associated protein 25A (SNAP-25A)], synaptotagmin 1, and complexin 1. However, other synaptic proteins could be added to the system and their function examined.

Early in vitro ensemble liposome studies showed that SNARE complex formation catalyzes lipid mixing (21). However, unlike neurotransmitter release, this process is constitutive and relatively slow (22, 23), even when preconditioning the system with a small C-terminal fragment (residues 49–96) of synaptobrevin (24), and, as mentioned above, these experiments did not measure content release, but rather lipid mixing. Likewise, a more recent single-vesicle experiment with SNAREs and full-length synaptotagmin 1 only monitored lipid mixing, and vesicles were observed in the presence of a constant Ca\(^{2+}\) concentration (25), so conclusions about Ca\(^{2+}\)-triggered content release cannot be drawn from this study. An ex vivo cell-based assay with “flipped” SNAREs and other factors demonstrated that SNAREs are capable of promoting content exchange (26), but fusion kinetics is much slower (approximate 10 min timescale) than that of neurotransmitter release, even in the presence of synaptotagmin and complexin (11). Only very few liposome-based experiments included a content-mixing indicator in studies of neuronal SNARE-dependent fusion (23, 27–29). In these studies, fusion was relatively slow (23, 27), very rare (28), or only vesicle leakage instead of fusion was observed (29). Moreover, these early content-mixing studies did not investigate Ca\(^{2+}\)-triggered fusion because synaptotagmin 1 and complexin were not present. Our results suggest that previous experiments that only used lipid-mixing indicators have to be repeated because lipid-mixing kinetics can be profoundly different from content-mixing kinetics.

The studies presented in this paper reveal insights in the molecular mechanism of Ca\(^{2+}\)-triggered synaptic vesicle fusion. We find that neuronal SNAREs alone do not efficiently overcome the activation barriers to achieve full fusion between membranes. These activation barriers are lowered upon Ca\(^{2+}\)-triggered fusion because synaptotagmin 1 and complexin are not present. Our results suggest that previous experiments that only used lipid-mixing indicators have to be repeated because lipid-mixing kinetics can be profoundly different from content-mixing kinetics.

**Results**

**Experimental Design.** To study the kinetics of single-vesicle fusion, we developed a triple-fluorescence-based single-vesicle assay that can distinguish between vesicle–vesicle interaction, hemifusion, and complete fusion. The system starts from a metastable state of interacting donor and acceptor vesicles that are formed during an incubation period (Fig. 1A). Ca\(^{2+}\) buffer is then injected in order to rapidly reach a defined Ca\(^{2+}\) concentration in the sample chamber. We monitor the instance of Ca\(^{2+}\) injection by detecting the fluorescence intensity of the cascade-blue dye that is part of synaptic vesicles in a self-quenched state. Donor vesicles that are bound to acceptor vesicles via SNARE protein–protein interactions are identified by the appearance of weak lipid dye fluorescence at a particular spot within the evanescent wave of the total internal reflection (TIR) microscope (Fig. S1). Lipid- or content-mixing events between single donor and acceptor vesicles that occur upon Ca\(^{2+}\) injection will produce stepwise fluorescence intensity increases for single donor vesicles due to dilution and concurrent depackaging of the dye. The simultaneous observation of lipid and content fluorescent dyes differentiates among characteristic events involving single interacting pairs of donor and acceptor vesicles, such as complete fusion (Fig. 1B and C, last stages), hemifusion (outer leaflet mixing, Fig. 1D, stage after Ca\(^{2+}\) injection), and interaction of vesicles without lipid mixing (Fig. 1C and D, stages prior to Ca\(^{2+}\) injection). Furthermore, instances of vesicle leakage are differentiated from fusion events (Fig. S2); such leakage events occurred very infrequently in our system, with less than approximately 0.01% probability—that low leakage rate was accomplished in part by using the content dye sulforhodamine B instead of calcine used in our earlier work (30).

**Characterization of Reconstituted Vesicles and Starting State of the System.** We performed a series of experiments to characterize the reconstituted donor (synaptobrevin, synaptotagmin 1) and acceptor (syntaxin/SNAP-25) vesicles. We used physiological compositions of donor (i.e., synaptic vesicle; ref. 31) and acceptor (i.e., active zone) membranes and physiological densities of the reconstituted proteins. The reconstituted vesicles exhibited a monodisperse size distribution as assessed by cryoelectron microscopy (cryo-EM) and dynamic light scattering (Figs. S3 and S4). The cryo-EM experiments also verified the integrity of the vesicles before and after fusion, and they indicate that the vesicles are unilamellar (Fig. S3 A–D).

We next determined the distribution of the number of protein molecules reconstituted into single vesicles by counting sequential photobleaching steps of dyes conjugated with proteins where only a fraction of the proteins were fluorescently labeled (Fig. S5). For correcting for the dilution factor and labeling efficiency, the average numbers of incorporated syntaxin, synaptobrevin, and synaptotagmin 1 molecules were 129 ± 11, 179 ± 14, and 28 ± 2 (for our 78-nm diameter vesicles), respectively. Thus, the reconstituted proteins have physiological densities as compared to observed distributions in synaptic vesicles with an average diameter of approximately 42 nm (32). More than 90% of syntaxin and synaptobrevin and 100% of synaptotagmin 1 molecules were properly reconstituted—that is, they point their cytosolic domains to the outside of the vesicles (Fig. S6). Purified syntaxin/SNAP-25 acceptor vesicles exhibited a 1:1 protein ratio (Fig. S64, left-most gel bands) suggesting that the majority of syntaxin molecules form productive 1:1 binary acceptor complexes with SNAP-25 (33).

Acceptor vesicles were then tethered to a pacified PEG-coated surface (34, 35) (Materials and Methods). Donor vesicles with content and lipid dyes were added and allowed to bind to acceptor vesicles via protein–protein interactions. After a defined incubation period, excess donor vesicles were removed by extensive washing and, depending on the experiment, including complexin in the buffer.

To characterize the starting state of our system, we performed a single-vesicle lipid-mixing assay with SNAREs alone, starting data acquisition right after addition of donor vesicles to the sample chamber (Fig. S7). Donor vesicles that had bound to acceptor vesicles underwent both fast and slow lipid-mixing processes (i.e., hemifusion) with ca. 20% probability during the 500-s observation period of this experiment (without Ca\(^{2+}\)). We interpret the fast process as instances of donor–acceptor vesicle pairs where a small number of trans SNARE complexes spontaneously form and trigger lipid mixing. The slower process could be related to diffusion of SNARE proteins in the vesicle membranes to form an encounter complex, followed by protein folding of a trans SNARE complex which is on the same order of magnitude as the slow lipid-mixing process that we observe (24, 36). The remaining ca. 80% of vesicles are simply interacting via trans SNARE com-
plexes, but without lipid exchange. In all subsequent experiments, we used a sufficiently long second incubation period (30 min) after removing excess donor vesicles to ensure that such folding processes have completed and the amount of hemifusion (approximately 20% of the vesicle pairs) has reached a plateau. We generally observe only one donor vesicle bound to a single acceptor vesicle based on the measured subsequent lipid and content-mixing events; very rare instances of multiple content-mixing events were excluded from the analysis. Thus, our system has a well-defined starting state of donor vesicles that are bound to acceptor vesicles, akin to the readily releasable pool of primed synaptic vesicles at the active zone of a synapse (20).

**Observation of Fast Complete Fusion upon Ca\(^{2+}\) Injection.** We injected Ca\(^{2+}\) into the sample chamber, starting from a set of single donor vesicles (containing both synaptobrevin and synaptotagmin 1) that are interacting with single acceptor vesicles (containing syntaxin/SNAP-25 acceptor complexes) in the presence of complexin (referred to as the “full system” in the following, although other factors are also important for synaptic vesicle fusion). The content and lipid dye fluorescence intensities rapidly increased for many vesicle pair spots upon Ca\(^{2+}\) injection (Movie S1), caused by dequenching of the dyes due to their respective mixing processes. Representative fluorescence intensity traces from single vesicles are shown in Fig. 2 A–C, revealing considerable heterogeneity of individual fusion pathways upon Ca\(^{2+}\) triggering. Content-mixing histogram analysis of all these individual traces revealed a rapid transition from approximately zero fusion to maximum fusion within one time bin (200 ms) upon injection of Ca\(^{2+}\) (i.e., with a rise time (i.e., the characteristic time from zero fusion to maximum fusion) that is faster than the time resolution of our setup (Fig. 2D, Left) (in fact, the time to reach 50% of the peak has to be considerably less than 0.1 s because one would otherwise observe two time bins to reach the maximum value). This rapid onset of fusion is followed by a biphasic decay that could be fitted to a double exponential function (Fig. 2D, Left, black line). The overwhelming contribution is from a fast exponential decay with a time constant of 0.25 ± 0.04 s (SD), whereas the remaining, smaller contribution is from a slower decay with a time constant of 1.6 ± 0.9 s (SD). Over the course of the observation period, a majority population (61 ± 4%) underwent complete

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**Fig. 1.** Experimental design of the single-vesicle content and lipid-mixing system. (A) Acceptor vesicles containing syntaxin (red) complexed with SNAP-25 (green) are tethered to a PEG-coated glass surface via biotin-neutravidin interactions. Protein-free vesicles are added to fill the voids between the acceptor vesicles (not shown for clarity). Donor vesicles containing synaptobrevin (black) and synaptotagmin 1 (blue) are labeled with content indicator fluorescent dyes (light and dark green), and lipid analog fluorescent dyes (gray and red). Both fluorescent dyes are initially self-quenched. Donor vesicles are added to the sample chamber (the incubation period actually consists of two stages, see Materials and Methods) in the presence or absence of complexin (yellow). Donor vesicles that are interacting with acceptor vesicles are identified by the appearance of weak lipid dye fluorescence spots in the evanescent wave. After the incubation period, a Ca\(^{2+}\) solution at a specified concentration is rapidly injected into the sample chamber, effectively replacing the sample volume 20 times (Materials and Methods). The arrival of the Ca\(^{2+}\) solution at a specified concentration is rapidly injected into the sample chamber, effectively replacing the sample volume 20 times (Materials and Methods). The arrival of the Ca\(^{2+}\) solution in the evanescent wave is monitored by the fluorescence intensity of the cascade-blue dye that is part of the injected Ca\(^{2+}\) solution. (B) Expected lipid dye fluorescence intensity (red) and content dye fluorescence intensity (green) time traces for an immediate content-mixing event starting from a hemifused state—i.e., with the outer leaflets merged—prior to Ca\(^{2+}\) injection. Upon Ca\(^{2+}\) injection, the inner leaflets merge and complete fusion occurs. (C) Expected fluorescence intensity time traces for a delayed content-mixing event. Upon Ca\(^{2+}\) injection, the outer leaflets of interacting donor and acceptor vesicles merge (Upper). After a time delay, the inner leaflets are also merging, and complete fusion occurs, resulting in the expected fluorescence intensity time traces shown in the lower panel. (D) Expected fluorescence intensity time traces for a hemifusion event. Upon Ca\(^{2+}\) injection, the outer leaflets of interacting donor and acceptor vesicles merge without undergoing complete fusion during the observation time period (Upper), resulting in the expected time trace shown in the lower panel.
fusion, and minority populations exhibited hemifusion only (14 ± 4%) or no change in fluorescence intensity (25 ± 9%).

In contrast to the full system, there is only little complete fusion upon calcium injection with neuronal SNAREs alone (i.e., without synaptotagmin 1 and complexin), followed by a single exponential decay of fusion with a slow time constant of 3.2 ± 1.4 s (SD) (Fig. 2E, Left and Movie S2). Only 20 ± 2% of the interacting vesicles exhibit complete fusion over the course of the observation period of 50 s and an additional 18 ± 4% of the interacting vesicles underwent hemifusion, but no complete fusion, during the observation period. The content-mixing histogram also provides evidence for a steady background fusion rate independent of calcium after calcium injection (approximately 0.2% of interacting vesicles per second, based on the limiting value that the fitted exponential function reaches for large times). The peak of the lipid-mixing histogram is about 10-fold higher than that of the content-mixing histogram (Fig. 2E, Right). Thus, most of this lipid-mixing peak relates to hemifusion-only events, and about half of these hemifusion events are followed by delayed complete fusion. The calcium-triggered fusion and lipid-mixing events that we observe for neuronal SNAREs alone are likely due to interactions between calcium and phosphatidylinositol 4,5-bisphosphate (PIP_2)-containing membranes (37) of the acceptor vesicles.

**Cooperativity of Calcium-Triggered Fusion.** To examine the calcium dependence of triggered release, we performed a series of single-vesicle fusion assays as a function of concentration of the injected calcium buffer. Fig. 3A (green dots) shows the rapid burst magnitude (as defined in Materials and Methods) vs. injected calcium concentration with neuronal SNAREs, synaptotagmin 1, and complexin. The curve can be well fit to a Hill function (Fig. 3A, Hill coefficient ca. 4) and calcium affinity (K_d ~ 3 mM) are statistically similar to those of the full system. Thus, synaptotagmin 1 molecules and their interactions with SNAREs and membranes produce a cooperative behavior as a function of calcium concentration. Complexin is therefore not required for the cooperativity of calcium triggering, consistent with the apparent lack of a calcium binding site for complexin.

There are a total of five possible calcium binding sites in the soluble C2 domains of synaptotagmin 1 that produce four intrinsic calcium binding affinities of 50, 140, 490 μM, and 3.1 mM as assessed by isothermal titration calorimetry at 25°C (38). Although synaptotagmin 1 binds to anionic membranes with micromolar affinity, and PIP_2 further enhances this binding (38), membrane binding itself may not be sufficient to trigger fusion. The millimolar calcium concentration required in our system implies that all calcium binding sites are occupied. We conclude that other factors missing in our assay must act in concert with SNAREs, synaptotagmin, and complexin in order to explain the different calcium range that is observed for action potential induced neurotransmitter release.
mitter release, starting at a few micromolar and saturating at 100 μM (39, 40).

Role of Synaptotagmin-1. We substituted synaptotagmin 1 with a mutant that disrupts the Ca$^{2+}$-binding site of the C2B domain of synaptotagmin 1 (D309A, D363A, and D365A, referred to as Syt1-C2BM); this mutant decreases the Ca$^{2+}$-dependent interaction of synaptotagmin 1 with anionic membranes and abolishes synchronous excitatory and inhibitory postsynaptic responses (41). For the Syt1-C2BM mutant, the rapid burst magnitude is reduced by a factor of approximately 5 (Fig. 4 A and I) as compared with the rate of the system containing wild-type synaptotagmin 1. We obtained a good fit to the content-mixing histogram with a single exponential decay function, suggesting that the underlying slow fusion process is similar to neuronal SNAREs alone. In contrast, both the total fusion occurrence over the entire observation period (Fig. 4 J) and the vesicle interaction efficiency (Fig. 4 K) were similar to those of the system with wild-type synaptotagmin 1. Thus, the Syt1-C2BM mutation has primarily kinetic consequences: It greatly reduces the rapid burst by delaying fusion events upon Ca$^{2+}$ injection.

Complete removal of synaptotagmin has an even more pronounced effect by essentially abolishing the rapid burst (Fig. 4 E and I), but also reducing the overall fusion occurrence (Fig. 4 J), while only slightly lowering the interaction efficiency (Fig. 4 K). These observed effects on fusion kinetics correlate well with the abolishment of fast synchronous release upon synaptotagmin 1 knockout and the poor rescue with the Syt1-C2BM mutant (41). Likewise, the lack of an effect on the interaction efficiency between donor and acceptor vesicles is reminiscent of the lack of an effect on the readily releasable pool of primed synaptic vesicles in synaptotagmin 1 knockout mice (41).

Role of Complexin. We investigated the role of complexin by replacing it with the mutant Cpx4M (R48A, R59A, K69A, Y70A) that reduces binding to the SNARE complex (10). As with wild-type complexin, there is a rapid fusion burst upon Ca$^{2+}$ triggering, but the magnitude is greatly reduced compared to that of the full system (Fig. 4 B and I). In contrast, the total fusion occurrence during the entire observation period with Cpx4M is statistically the same as that of the full system with wild-type complexin (Fig. 4 J). Interestingly, the vesicle interaction efficiency is also reduced (by a factor of approximately 3) compared to the full system with wild-type complexin (Fig. 4 K).

We next tested the effect of removal of complexin on fast Ca$^{2+}$-triggered fusion (i.e., only SNAREs and synaptotagmin 1 are present in the system). Despite the absence of complexin, there is a rapid burst upon Ca$^{2+}$ injection (Fig. 4 C), although the rapid burst magnitude is significantly reduced compared to that of the full system (Fig. 4 H and I). As with the full system, the substitution of wild-type synaptotagmin 1 with the Syt1-C2BM mutant essentially eliminates the rapid burst (Fig. 4 D and I), resulting in behavior similar to with neuronal SNAREs alone.

The total fusion occurrence over the entire observation period is similar to that of full system when complexin is absent (Fig. 4 J), whereas the vesicle interaction efficiency is greatly reduced (Fig. 4 K). As with the Cpx4M mutant, the absence of complexin slows Ca$^{2+}$-triggered fusion kinetics and lowers the interaction efficiency between donor and acceptor vesicles. Thus, our system mimics the reduction of fast synchronous release in complexin knockout and rescue studies of cortical rat neurons (41). Furthermore, the reduced vesicle interaction efficiency in the absence of complexin correlates with the reduction of the readily releasable pool of primed synaptic vesicles in complexin 1 knockout studies in rat cortical neurons (42). The molecular basis of complexin’s function in enhancing the interaction between donor and acceptor vesicles may be explained by previous single-molecule studies: Interaction between complexin and syntaxin/SNAP-25 promotes folding of the flexible syntaxin/SNAP-25 complex, thereby making it more likely to interact efficiently with synaptobrevin (43).

Dependence on SNARE Complex Formation. As control, we disrupted trans SNARE complex formation by using the soluble cytoplasmic fragment of synaptobrevin, residues 1–96. When acceptor vesicles are incubated with excess of this fragment prior to the injection of donor vesicles into the sample chamber, it siphons away most available acceptor (syntaxin/SNAP-25) complexes. As a consequence, the vesicle interaction efficiency was reduced to approximately 14 ± 2 (SD)% (Fig. 4 K, right-most bar). For those vesicles that did interact, the total fusion occurrence over the entire observation period was dramatically reduced (Fig. 4 J), and no rapid fusion burst was observed (Fig. 4 G and I), compared to the full system. Thus, as expected, disruption of the ability to form trans SNARE complex greatly reduces vesicle interaction and essentially abolishes fusion.

Discussion

Single-Vesicle Content-Mixing System with Fast Ca$^{2+}$-Triggered Fusion Kinetics. Our single-vesicle content-mixing system exhibits fast complete fusion and cooperativity upon Ca$^{2+}$ injection (Figs. 2D and 34). Starting from interacting single donor and acceptor vesicle pairs, we monitored instances of content and lipid mixing. SNAREs, synaptotagmin 1, and complexin are all essential to obtain the most pronounced rapid burst of content mixing (Fig. 4). The burst has a rise time that is so fast that it cannot be resolved with our system, followed by a slower decay with a time constant of 250 ms. In analogy to our Ca$^{2+}$ delivery method, in vivo experiments that employ photolysis of caged Ca$^{2+}$ also produce a step-
wise increase of Ca\textsuperscript{2+} concentration in the axonal terminal (44, 45). These in vivo experiments revealed a very rapid rise for Ca\textsuperscript{2+} in the vesicle membrane (i.e., a rapid rise followed by a slower decay). The Ca\textsuperscript{2+} range for our system is different from that observed for action potential induced neurotransmitter release (approximately 1–100 \mu M) (39, 40), although the cooperativity (Hill coefficient ca. 4) of our system is physiological. It should be noted that the lipid-mixing-only assay reported in ref. 25 did not produce cooperative behavior (paradoxically, less fusion was observed at higher Ca\textsuperscript{2+} concentration).

The system used in our present study, most probably does not include all of the factors needed to reproduce the Ca\textsuperscript{2+} dose response and faster decay kinetics observed in vivo. For example, the proteins Munc18 (46), RIM (47), Munc13 (48), and Ca\textsuperscript{2+}-dependent activator protein for secretion (CAPS) (49) play important roles in exocytosis, and their molecular function could be assessed with our single-vesicle content-mixing system. It is remarkable, however, that the minimal system of SNAREs, synaptotagmin, and complexin qualitatively mimics effects on Ca\textsuperscript{2+}-triggered fast synchronous release (correlated with the observation of content mixing in our system) and the readily releasable pool of synaptic vesicles (correlated with the vesicle interaction efficiency in our system) for key mutants of synaptotagmin 1 and complexin observed by in vivo studies in cortical neurons (10, 41) (Fig. 4).

**Content Mixing Vs. Lipid Mixing.** Measuring just lipid mixing can be misleading: Although there is a burst of lipid mixing for neuronal SNAREs alone upon Ca\textsuperscript{2+} injection, the kinetics of content mixing (the quantity that is relevant for neurotransmitter release) is actually much slower for neuronal SNAREs alone compared...
to the full system (compare Fig. 2 D and E). Thus, our results indicate that an increase in lipid mixing cannot be reliably interpreted as complete fusion, in agreement with other studies on a variety of biological fusion processes (15–19). Even at a single-vesicle level, it is often not possible to detect two clearly defined increases in lipid mixing, resulting in the inability to distinguish between inner and outer leaflet mixing for such a single step instance. Although there is a more pronounced lipid-mixing peak for the full system compared to SNAREs alone, consistent with previous lipid-mixing liposome experiments (50, 51), this difference in lipid mixing does not reveal the dramatic difference in content-mixing kinetics (compare Fig. 2 D and E, Left). In addition, our system can distinguish individual instances of vesicle leakage from that of content release (Fig. S2); this is very difficult to discern by ensemble assays, especially because only a few leakage events could have a significant effect on the average fluorescence intensity. In any case, our system has a very low probability of vesicle leakage events (less than 0.01%). Thus, our single-vesicle content-mixing system is a major advance compared to previous in vitro approaches to study synaptic vesicle fusion. Many lipid-mixing-only experiments carried out over the past decade may need to be repeated with content-mixing indicators.

**Ca\(^{2+}\)**-Triggered Catalysis of SNARE-Dependent Fusion by Synaptotagmin. Fig. 5 is a qualitative summary of our findings in a framework of states and transitions between states in analogy to theories about the steps involved in biological membrane fusion (52). Neuronal SNAREs alone (i.e., without synaptotagmin and complexin) readily promote donor–acceptor vesicle interaction, and some lipid mixing, consistent with previous results (53). Yet, neuronal SNAREs alone do not efficiently produce fast Ca\(^{2+}\)-triggered complete fusion (Fig. 2E, Left). Using a different content-mixing assay, spontaneous complete fusion activity of neuronal SNAREs alone in the absence of Ca\(^{2+}\) was also found to be slow (23); in fact, endogenous fusion activity appears to be slower for neuronal SNAREs than for yeast SNAREs (54, 55). Thus, one can speculate that neuronal SNAREs have evolved to generate less power than yeast SNAREs to overcome the transition barriers for complete fusion between membranes.

The combination of synaptotagmin 1 and SNAREs effectively lowers the transition barriers for complete fusion between membranes upon Ca\(^{2+}\) injection (Fig. 5). Ca\(^{2+}\) binding to synaptotagmin is essential for this process because mutation of the Ca\(^{2+}\) binding site of the C2B domain of synaptotagmin 1 greatly reduces the rapid burst magnitude (Fig. 4I, consistent with its effect on fast synchronous neurotransmitter release (41). How could synaptotagmin 1 in concert with SNAREs drive fusion upon Ca\(^{2+}\) injection starting from a metastable state of interacting membranes? First, synaptotagmin has a profound effect on membranes and membrane interactions (56, 57), possibly destabilizing the membrane near a hemifusion stalk. Second, it might trigger protein conformational changes in SNAREs upon Ca\(^{2+}\) binding, as suggested by single-molecule studies of the interaction between synaptotagmins and SNARE complex (58, 59). Third, it might induce formation of additional trans SNARE complexes upon Ca\(^{2+}\) triggering that would provide the energy to drive the system to full fusion. Future developments of the single-vesicle fusion system should allow time-resolved studies of the molecular mechanism involved in this process.

**Enhancement of Ca\(^{2+}\)** Control by Complexin. Complexin increases the rapid fusion burst as well as vesicle interaction efficiency consistent with its activating function (Fig. 4 I and K). However, in the absence of complexin, some Ca\(^{2+}\)-triggered rapid fusion still occurs at higher Ca\(^{2+}\) concentrations (compare the green and purple lines in Fig. 3A). In contrast, at lower Ca\(^{2+}\), complexin had little effect. These results suggest that complexin’s activating characteristic (10, 11) is a manifestation of two separate effects: enhancement of the fast fusion burst (accompanied by reduction of “slow” or spontaneous fusion events) at higher Ca\(^{2+}\) concentration, and an enhancement of the membrane interaction efficiency. Moreover, complexin is not absolutely required to observe cooperative Ca\(^{2+}\)-triggered fusion—SNAREs and synaptotagmin 1 are sufficient and constitute a most minimal Ca\(^{2+}\)-triggered fusion system, albeit less efficiently than the presence of complexin. This conclusion could not be drawn in previous studies that only used lipid-mixing indicators. Such work concluded that neuronal SNAREs alone produce significant lipid-mixing activity on their own (also reproduced in our lipid-mixing studies, Figs. S7 and S8). Because lipid mixing was interpreted at the time as complete fusion, it was thought that some sort of fusion clamp must always exist to prevent full fusion in the absence of Ca\(^{2+}\). Our results now show that such a clamp is not absolutely required for neuronal SNAREs: Very little complete fusion is observed in the starting state of interacting vesicles until Ca\(^{2+}\) arrives, even in the absence of complexin (see Fig. 2D and E, Left, before Ca\(^{2+}\) injection). Along similar lines, the conclusions drawn from other lipid-mixing studies implying an arresting role of synaptotagmin before Ca\(^{2+}\) triggering (60) have to be revisited.

**Outlook.** Our single-vesicle content-mixing system provides a platform to study the role of various factors in Ca\(^{2+}\)-triggered synaptic vesicle fusion. The results presented in this paper demonstrate the power of the synthetic system: One can deliberately omit or manipulate components to determine which parts are essential and monitor what happens to the system when essential parts are missing. By directly observing individual protein–protein interactions in conjunction with single-vesicle content and lipid-mixing indicators, this system will provide a foundation to investigate the molecular mechanism of synaptic neurotransmitter release. We predict that a molecular understanding of this fundamental biological process is within reach.
Materials and Methods

Protein Expression and Purification. Expression of complexin and SNAP-25 in Escherichia coli. Recombinant full-length rat neuronal complexin 1 (simply referred to as complexin), the Cpx4M mutant of complexin (R48A, R59A, K69A, Y70A) (10), and the mutant of SNAP-25A (simply referred to as SNAP-25) (61) with all endogenous cysteine residues substituted with serine were subcloned into pET22B (Novagen, EMD chemicals) and expressed as N-terminal His6-tagged fusion proteins in BL21 (DE3) E. coli cells (Novagen, EMD chemicals). Cells were grown with 35 mg/mL kanamycin in terrific broth media. Cell induction was performed with 1 mM IPTG at an optical density A600 = 1.0 for 3 h at 37 °C.

Expression of SNAP-25 in Spodoptera frugiperda (Sf9) cells. By virtue of the removal of the endogenous cysteine residues and expression in E. coli, SNAP-25 is not palmitoylated. We therefore also expressed SNAP-25 in Sf9 cells using a similar protocol as for full-length synaptotagmin 1, which resulted in palmitoylation of the endogenous SNAP-25 residues as assessed by mass spectroscopy. We observed similar rapid bursts and decay time constants with and without SNAP-25 palmitoylation (compare the content-mixing histograms in Fig. 2D and Fig. S9). However, due to significantly lower yield of SNAP-25 expressed in Sf9 cells, we therefore used SNAP-25 expressed in E. coli for most experiments.

Expression of full-length syntaxin and synaptobrevin in E. Coli. We modified the full-length rat neuronal syntaxin-1A (S193C, C271S, C272S) expression construct used in previous work (61) by replacing the N-terminal His6-tag threonin cleavage site with a tobacco etch virus (TEV) protease site to prevent secondary cleavage of syntaxin by thrombin (this modified construct is simply referred to as syntaxin). This mutant of full-length syntaxin-1A, and full-length wild-type (and the S28C mutant of) rat neuronal synapto-}

Vesicle Composition and Reconstitution of Syntaxin, Synaptobrevin, and Synaptotagmin 1. Proteins were reconstituted into vesicles with a detergent depletion method (63). Acceptor (syntaxin/SNAP-25) vesicles were prepared from Brain Total Lipid Extract, supplemented with 20 mol% Cholesterol, 3.5 mol% PIP2, and 1 mol% biotinylated phosphatidylethanolamine (PE) (all lipids from Avanti Polar Lipids). Donor (synaptobrevin/synaptotagmin 1) vesicles were prepared as detergent-depleted SDS gel electrophoresis. Both glycosylated and nonglycosylated proteins were present and used in all experiments. We tested the glycosylation state by using a tunicamycin assay (addition of tunicamycin during Sf9 cell expression resulting in nonglycosylated protein, Fig. S6).

The cyttoplasmic domain of rat synaptobrevin-2 (1–96) was expressed and purified as previously described (61).

Expression of full-length synaptotagmin (Sf9) cells. A construct of rat neuronal full-length synaptotagmin 1 (residues 1–421) with a C-terminal His6-tag thrombin cleavage site cloned into the pENTR/TEV/D-TOPO entry vector (Invitrogen), using PCR primers synaptotagmin 1 TOPO forward 5′-gattctagtgcagctcagctg3′ and synaptotagmin 1 TOPO reverse 5′-tcaatgatgatggtgatgatggtggtg 3′ for directional cloning into the TOPO entry vector (based on pENTR directional TOPO cloning kit). The recombinant entry vector was used to perform the LR Clonase™ II reaction to transfer the synaptotagmin 1 full-length gene in the baculodirect C-terminal destination vector (Invitrogen). The TOPO constructs for the Syt1-C2BM mutant of synaptotagmin 1 (D309A, D363A, D365A) (41) and the cytoine mutant of synaptotagmin 1 (C745, C755, C775, C795, C825) were synthesized by GENEART AG.

Recombinant full-length rat neuronal synaptotagmin 1, the Syt1-C2BM mutant, and the cytoine mutant were expressed as C-terminal His6-tagged fusion proteins in Sf9 insect cells (Invitrogen). Sf9 cells were grown in tetra-nitrothiane-fumurate hydrate (TMN-FH) media. Cell induction was performed with 1 mM IPTG at an optical density A600 = 1.0 for 3 h at 37 °C.

Purification of complexin and SNAP-25. His6-tagged fusion proteins were purified by Ni2+–nitrilotriacetic acid (NTA) sepharose (Qiagen) affinity chromatography from crude cell lysate. The bound proteins were washed with 50 mM imidazole concentration in a buffer containing 20 mM Hepes (pH 7.4), 300 mM NaCl, and 2 mM DTT, and then eluted in 400 mM imidazole. Eluted proteins were further purified by size-exclusion chromatography (AKTA, GE Healthcare) using a Superdex 200 10/300 column (GE Healthcare) in buffer containing 20 mM Hepes (pH 7.4), 100 mM NaCl, and 4 mM DTT; the elution profiles consisted of a dominant, symmetric peak for each of the proteins. The elution profiles peaked at 0.7 and 0.8 for the donor and acceptor proteins, respectively, at a detergent concentration was below the critical micelle concentration; the detergent concentration was below the critical micelle concentration; the solutions were purified with a CL4B column and diazoylated with Bio-beads SM2 (Bio-Rad) in protein-free Vesicle Buffer (20 mM Hepes (pH 7.4), 90 mM NaCl, 20 μM EGTA, 1% 2-mercaptoethanol). Donor (synaptobrevin/synaptotagmin 1) vesicles were formed in the presence of 50 mM sulforhodamine B (Invitrogen), prior to SEC and dialysis. Size distributions of reconstituted vesicles were characterized with quasi-elastic light scattering (Nano-ZS90, Malvern Instruments) and cryoelectron microscopy (Figs. S3 and S4), revealing an average diameter of 78 nm. The effect of two different vesicle diameters (30 and 100 nm) on vesicle interaction (“vesicle-vesicle binding”) and lipid-mixing kinetics was found to be relatively small (64), suggesting that much larger acceptor vesicle diameters (as mimicking the active zone geometry) would not significantly affect our
results. The proper insertion of proteins into vesicles was assessed by partial proteolysis (Fig. S6).

Surface Preparation for Single-Vesicle Experiments. Cover and slide glasses were thoroughly rinsed and sonicated in acetone and methanol repeatedly more than 10 times over several days. 3% vol/vol solution of 3-aminopropyl-triethoxysilane (Sigma-Aldrich) in acetone was applied to cover glasses for 30 min. Subsequently, surfaces were coated with a PEGbiotin/PEG mixture consisting of PEG-SCM 5000 and Bio-PEG-SCM 5000 (both Laysan Bio) at a 9:1 ratio, sulfate buffer (0.5 M potassium sulfate, 50 mM sodium phosphate), and incubated for 2 h. Residual PEG solution on the surfaces was removed with a large excess of deionized water (Millipore).

Protein Number Distributions in Single Vesicles. The single-cysteine mutants of full-length syntaxin, synaptobrevin, and synaptotagmin 1 were labeled with Cy3-maleimide (Amersham Biosciences). Ni-NiTA bound single-cysteine containing mutants of full-length synaptotagmin 1, synaptobrevin, and syntaxin were washed with 300 mM NaCl, 100 μM Tris(2-carboxyethyl)phosphine, 110 mM OG, and 20 mM sodium phosphate (pH 7.4) or 20 mM Hepes (pH 7.4) and incubated with Cy3-maleimide (Amersham Biosciences) for 2 h at room temperature, followed by overnight incubation at 4 °C. Excessive washing of the bound protein was performed to remove free dye, and labeled protein was then eluted in 500 mM NaCl, 2 mM DTT, 110 mM OG, and 20 mM sodium phosphate (pH 7.4) or 20 mM Hepes (pH 7.4). Eluted proteins were further purified by SEC (AKTA, GE Healthcare) using a Superdex 200 10/300 column (GE Healthcare) in buffer containing 20 mM sodium phosphate or Hepes (pH 7.4), 100 mM NaCl, and 4 mM DTT. The labeling efficiency was 80% for the full-length syntaxin, 90% for the single-cysteine mutant of full-length synaptobrevin, and 12% for the single-cysteine mutant of synaptotagmin 1 as determined by UV-visible absorption of the dyes at 535 and 635 nm and the protein at 280 nm (A280), and their respective extinction coefficients. The A280 value stayed within 3% error upon denaturation of the samples in 6 M GuHCL.

Labeled proteins were serially diluted with unlabeled proteins and reconstituted into vesicles. For the donor (synaptobrevin, synaptotagmin 1) vesicles, 1 mol% biotinylated-PE was added into the original lipid mixture to enable surface immobilization. The protein-reconstituted vesicles were immobilized on PEG-coated glass surfaces similar to previously described methods and imaged by single-vesicle TIR fluorescence microscopy (see below) until the signal disappeared by photobleaching. Among several ratios of labeled/unlabeled protein mixtures, we selected one that showed distinct fluorescence intensity photobleaching steps for most of the vesicles and determined the distribution of the number of labeled proteins in single vesicles (Fig. S5). The average number and distribution of labeled proteins in single vesicles was obtained by fitting the distribution with a logarithmic function. Using the known dilution factors of labeled and unlabeled proteins, and labeling efficiencies, the distribution of the total number of reconstituted proteins was calculated from the measured number of labeled proteins in single vesicles. The reconstitution efficiencies were 54.3%, 75.4%, and 53.2%, respectively, calculated by comparison of the measured protein to lipid ratio to that in the initial mixture. The protein number (Fig. S5) and vesicle size distributions (Figs. S3 and S4) are generally homogenous, an important prerequisite for in vitro fusion studies (65).

Combined Single-Vesicle Interaction, Content, and Lipid-Mixing Assay. A PEG-coated glass surface was incubated with neutravidin solution (50 μg/mL, 20 mM, 90 mM NaCl) for 15 min. Acceptor (syntaxin/SNAP-25) vesicles were immobilized on the reconstituting surface similar to previously described methods, and excess vesicles were removed by thoroughly washing with Vescie Buffer. Protein-free vesicles with the same lipid composition as donor vesicles were reconstituted with a large excess of deionized water (Millipore).

Analysis of Single-Vesicle Fluorescence Intensity Time Traces. The spots corresponding to interacted vesicles were identified and background-corrected with a customized program written for IDL graphic system (ITT Visual Information Solutions) (66). For each pixel, the mean value of the neighboring background pixels was subtracted from the pixel. The fluorescence intensity was due to dilution and concurrent dequenching of the dyes. We can rule out a hypothetical scenario where some donor vesicles would simply move closer to the surface within the evanescent wave because we ensured that there is no nonspecific interaction between donor vesicles and the surface.

Consecutive fluorescence images were acquired with exposure times of 200 and 500 ms unless noted otherwise. Vescie scattering prevented data acquisition at shorter exposure times due to background noise. Content-assimilating histograms were calculated with 1-s binning for 1-s binning, for 1-s binning, consecutive 500-ms bins were combined into one bin. We used 1-s binning with 500-ms exposure times for some of the comparisons in order to reduce noise. At least three independent sets of experiments were performed, each with freshly prepared proteins and vesicles, and representative histograms are taken from one of the corresponding experiments unless noted otherwise. All histograms were normalized with respect to the number of interacting vesicles for the particular experiment.

Exponential decay functions were fitted to the histograms using IGOR Pro (WaveMetrics). The goodness of fit was evaluated with χ2 in order to determine how many fitting parameters are needed. The time constants of the exponential fits to histograms calculated with 200-ms and 1-s binning are similar (Figs. 2D and 4H), so for some experiments, we choose a histogram binning of 500 ms or 1 s. In agreement with our observations, computer simulations of an exponential decay with greatly exaggerated noise showed that, for a given time bin, time constants of one-third of the time bin can be reliably determined.

For quantitative comparison of the content-mixing histograms for different experiments, we calculated the following four quantities. The vesicle interaction number is the number of donor vesicles that are interacting with acceptor vesicles, as determined by the number of fluorescent lipid dye spots of many of them are clearly visible before Ca2+ injection, but some of them are weak, so we confirmed these weak spots as interacting vesicles by a subsequent increase in fluorescence after Ca2+ injection). This quantity can be compared among different experiments in Fig. 4 because the concentration of acceptor vesicles on the surface and the incubation times are identical in

all experiments. Furthermore, we normalized the vesicle interaction number relative to the full system (we refer to this as the vesicle interaction efficiency). The overall fusion occurrence is the number of donor vesicles that completely fuse with acceptor vesicles during the entire observation period (50 s) divided by the vesicle interaction number. The rapid burst amplitude is the number of donor vesicles that completely fuse with acceptor vesicles during the first 1 s after Ca2+ injection divided by the vesicle interaction number.

Standard deviations for these quantities were obtained from multiple sets (at least three) of experiments, each with freshly prepared proteins and vesicles.

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Supporting Information

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Fig. S1. Microscope setup. A three-color, objective-based total internal reflection (TIR) instrument was built using a Nikon TE2000U inverted microscope (Nikon). The optical paths of two diode lasers (cube 640 and 405 nm, Coherent) and a diode-pumped solid state laser (Excelsior 532 nm, Spectra-Physics, Newport) were combined with dichroic mirrors and delivered to the back focal plane of the objective (Nikon Apo TIR 100×, 1.49 N.A.). A converging lens (focal length $f = 300$ mm), located conjugate to the sample plane, allowed translation of the laser along the $x$ and $y$ axes. A custom-built, triple-view imaging path between the microscope and EM-CCD cameras (iXon EM+ DU-897, Andor Technology) separated the fluorescence emission into three spatially and temporally identical, but spectrally distinct, images of single vesicles. The fluorescence emissions from sulforhodamin and 1,1'-dioctadecyl-3,3',3',3' tetramethylindodicarbocyanine were detected through FF01-562/40 (Semrock) and HQ700/75m (Chroma Technology) optical bandpass filters, respectively. All experiments were carried out at ambient temperature (ca. 20°C). Images were acquired in kinetic mode with a frame rate of 5 or 2 Hz. The size of the point spread function (PSF) is $s_{PSF} = 0.44l/(2N.A.)$, the rms size of the Airy disk-shaped microscope PSF. In this setup, $s_{PSF} = 99$ nm, using $\lambda = 670$ nm and the N.A. = 1.49.

Fig. S2. Content- and lipid-mixing time traces of a leaking vesicle. The particular event shown in this figure corresponds to one of the spots in Movie S1. Green and red lines show the content and lipid dye fluorescence intensity over time, respectively. After a rapid jump at approximately 0.2 s, the content dye fluorescence intensity decays rapidly within one time bin, consistent with three-dimensional diffusion of the dye due to leakage from the donor vesicle. The fluorescence intensity of the 1,1'-dioctadecyl-3,3',3',3' tetramethylindodicarbocyanine lipid dye increases due to reduction of the self-quenching effect by lipid mixing (hemifusion) of the donor and acceptor vesicles upon $Ca^{2+}$ injection at $t = 0$. Instances of vesicle leakage are very rarely (ca. 0.01%) observed in our experiments.
Fig. S3. Characterization of vesicles by cryo-EM. (A–D) Showing representative regions of cryo-EM images of (A) individual syntaxin/synaptobrevin-2, syntaxin-1A, and synaptosomal-associated protein 25A (SNAP-25A) vesicles (light-gray circles with dark borders with approximately 70 nm diameter), (B) individual synaptobrevin/synaptotagmin 1 vesicles, (C) a mixture of syntaxin/SNAP-25 and synaptobrevin/synaptotagmin 1 vesicles without Ca\(^{2+}\), and (D) a mixture of syntaxin/SNAP-25 and synaptobrevin/synaptotagmin 1 vesicles after multiple rounds of fusion in the presence of Ca\(^{2+}\). We used a ninefold excess of syntaxin/SNAP-25 vesicles in order to promote multiple rounds of fusion, and, consequently, a more pronounced increase in vesicle diameters (compare C and D) (note that these experiments were carried out in solution). (Scale bars: 200 nm.) The dark elongated features are the lacy carbon-coated polyvinyl formal film and the dark small spots and fuzzy features are ice crystals. Samples for cryo-EM were prepared using a Vitrobot (FEI) at 100% relative humidity. Four microliters of vesicle solutions were incubated on a glow-discharged lacey Formvar/carbon 300 mesh copper grid (Ted Pella) before blotting and plunging into liquid ethane. The frozen-hydrated specimens were subsequently observed at liquid nitrogen temperature in a Tecnai 12 electron microscope (FEI) operated at 120 kV, under low dose conditions. Images were collected on a MSC794 CCD camera (Gatan) at a nominal magnification of 18,500, resulting in a 0.8 nm per pixel scale at the specimen level. (E and F) Size distribution of (E) individual synaptobrevin (SB)/synaptotagmin 1 (Syt1) vesicles and (F) individual syntaxin (SX)/SNAP-25 (SN25) vesicles obtained by analysis of the cryo-EM images. Red lines indicate a lognormal function fit.
Fig. S4. Characterization of vesicles by light scattering. (A and B) Size distributions of (A) synaptobrevin (SB)/synaptotagmin 1 (Syt1), and (B) syntaxin (SX)/SNAP-25 (SN25) vesicles determined by dynamic light scattering. The average sizes and distributions obtained from cryo-EM (Fig. S3) and light scattering are similar.

Fig. S5. Protein number distributions in single vesicles. (A–C) Histograms of the number of labeled synaptobrevin (SB), syntaxin (SX), and synatotagmin 1 (Syt1) molecules in single vesicles, respectively. The same number densities were used for all experiments. The red star indicates which protein is labeled. In order to avoid partial self-quenching if too many dyes are present in a single vesicle, and in order to enable counting of stepwise decreases in fluorescence intensity by photobleaching events, labeled proteins were diluted with nonlabeled proteins at selected ratios (1:62.5, 1:50, and 1:8.3, for A, B, and C, respectively) and reconstituted into the vesicles. We calculated the total number of reconstituted proteins per single vesicle by multiplying the dilution factors with the measured number of labeled proteins and taking into account the labeling efficiencies (see Materials and Methods). This method worked for up to ca. 10 dyes in a particular vesicle; for the few vesicles with more than 10 dyes, the number was estimated by the fluorescence intensity of the spot. (D) Representative time traces of labeled synaptobrevin reconstituted into single vesicles. Labeled synaptobrevin molecules were diluted with unlabeled molecules at a ratio of 1:62.5. The time interval for image acquisition was 2 s. Stepwise decreases in the fluorescence intensity time traces indicate photobleaching events. The upper-right trace shows an increase in fluorescence intensity at 150 s, indicating sudden emission of a single dye after emerging from a dark state.
mixing of vesicles is similar to that reported by previous lipid-mixing experiments (1). We estimate that, after a 30 min incubation time, the system is within 1% of reaching a lipid-mixing plateau. In our system, the timescale of SNARE-mediated vesicle docking is comparable to that observed in the experiments of Kyoung et al. (2).

Fig. S7. Single-vesicle lipid mixing induced by neuronal SNAREs alone. Showing a histogram (red) of lipid-mixing occurrences as a function of the delay time after an instance of single vesicle–vesicle interaction. Acceptor (syntaxin/SNAP-25) vesicles were immobilized to the PEG-coated glass surface using the same method as described for the combined single-vesicle content and lipid-mixing assay. Similarly, donor vesicles containing only synaptobrevin molecules were labeled with 3.5 mol % 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI). The fluorescence intensity of DiI dyes from single donor vesicles was imaged over a 500-s observation period right after addition of the donor vesicles to the sample chamber. The time differences from the instance of single vesicle docking (first incremental step of DiI fluorescence intensity) and subsequent dequenching events (subsequent incremental increases of DiI fluorescence intensity) of single vesicles were analyzed from single-vesicle fluorescence intensity time traces and used to build a histogram and cumulative distribution function. An initial increase of DiI fluorescence intensity is indicative of vesicle interaction. A second increase in DiI fluorescence intensity is indicative of donor/acceptor lipid mixing. Only approximately 25% of the docked vesicles underwent lipid mixing even after a long incubation period of 500 s. All experiments were performed in Vesicle Buffer without CaCl₂.

Fig. S8. Lipid-mixing histograms corresponding to Fig. 4. (A) Representative lipid-mixing histogram with SNAREs (SB, synaptobrevin; SX, syntaxin; SN25, SNAP-25), the mutant of synaptotagmin 1 (Syt1) that disrupts Ca\(^{2+}\) binding to the C2B domain (Syt1-C2BM), and complexin (Cpx). The black line is a fit to the histogram \(f(t) = 0.19 + 100.79 \cdot e^{-1.69t}\). (B) Representative lipid-mixing histogram with SNAREs, Syt1, and the mutant of complexin that disrupts SNARE complex binding (Cpx4M). The black line is a fit to the histogram \(f(t) = 0.09 + 122.98 \cdot e^{-2.53t} + 16.07 \cdot e^{-0.60t}\). (C) Representative lipid-mixing histogram with SNAREs and Syt1. The black line is a fit to the histogram \(f(t) = 0.01 + 278.2 \cdot e^{-1.00t} + 6.44 \cdot e^{-0.45t}\). (D) Representative lipid-mixing histogram with SNAREs and the Syt1-C2BM mutant. The black line is a fit to the histogram \(f(t) = 0.02 + 125.42 \cdot e^{-1.58t} + 3.61 \cdot e^{-2.15t}\). (E) Representative lipid-mixing histogram with SNAREs and Cpx. The black line is a fit to the histogram \(f(t) = 0.07 + 55.15 \cdot e^{-2.42t} + 8.08 \cdot e^{-0.66t}\). (F) Representative lipid-mixing histogram with SNAREs and mutant Cpx4M. The black line is a fit to the histogram \(f(t) = 0.07 + 55.15 \cdot e^{-2.42t} + 8.08 \cdot e^{-0.66t}\). (G) Representative lipid-mixing histogram with SNAREs, the cytoplasmic domain of synaptobrevin (Sol-SB), Syt1, and Cpx. The black line is a fit to the histogram \(f(t) = 0.01 + 18.78 \cdot e^{-0.70t}\). (H) Representative lipid-mixing diagram for the full system with wild-type proteins, similar to Fig. 2D, Right, except for a 0.5-s exposure time for each image and 1-s binning for calculating the histogram. The black line is a fit to the histogram \(f(t) = 0.01 + 559.36 \cdot e^{-2.42t} + 5.30 \cdot e^{-0.23t}\). All are normalized with respect to the number of interacting vesicles. Time \(t = 0\) corresponds to the instance of Ca\(^{2+}\) injection as determined by the appearance of cascade-blue fluorescence.
Fig. S9. Content-mixing histogram with palmitoylated SNAP-25. Showing fast Ca\(^{2+}\)-triggered fusion with palmitoylated SNAP-25 for the full system (i.e., in the presence of SNAREs, synaptotagmin 1, and complexin). The content-mixing histogram was obtained from the combination of five individual experiments (n = 168). The histogram was normalized with respect to the total number of events in the combined histogram. The black line is a fit to the histogram 
\[ f(t) = 0.03 + 31.1 \times e^{-4.2t} + 7.4 \times e^{-0.7t}. \] Time constants were similar to that of the system with nonpalmitoylated SNAP-25 (Fig. 2D, Left).

Movie S1 Content and lipid-mixing events with SNAREs, synaptotagmin 1, and complexin. Shown are the fields of view (63 × 63 μm) of the fluorescence intensities of the content-mixing (green, Left) and lipid-mixing dyes (red, Right). The time interval is 200 ms. The counter in the lower-right corner is in seconds. The yellow trace indicates the fluorescence intensity of the cascade-blue dye that is a component of the injected Ca\(^{2+}\) solution, so the increase in the fluorescence intensity at 1.6 s indicates Ca\(^{2+}\) injection. Note that the colors in the video are chosen for illustration.

Movie S1 (MOV)

Movie S2 Content and lipid-mixing events with SNAREs alone. The time interval is 500 ms and the Ca\(^{2+}\) injection occurs at 11.5 s. Fields of view and the yellow trace are similar to Movie S1.

Movie S2 (MOV)
In vitro system capable of differentiating fast Ca\(^{2+}\)-triggered content mixing from lipid exchange for mechanistic studies of neurotransmitter release

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**AUTHOR SUMMARY**

Neuronal communication is made possible by the release of neurotransmitters, which in turn depends on the fusion of neurotransmitter-laden synaptic vesicles at the axonal ends of nerve cells. Synthetic vesicle fusion is triggered by an influx of Ca\(^{2+}\) ions into the neuron upon depolarization, an event that occurs during neurotransmission.

Neurotransmitter release is quantized, sometimes involving tens of individual synaptic fusion events. The process of individual synaptic vesicle fusion is in turn controlled by a handful of proteins, such as the SNARE proteins (1–3), the Ca\(^{2+}\) sensor synaptotagmin 1, and the modulator complexin. Thus, neurotransmitter release is a biological phenomenon controlled by a few individual molecules. The understanding of the underlying molecular mechanisms requires methods that are inherently capable of observing single vesicles and molecules (4).

Ideally, observations of single vesicles and molecules would be performed in live neurons. Although progress for such studies has been made, they currently provide limited information, largely because the genetic manipulations or labeling techniques used may not provide the spatial and time resolution required for studying the dynamics of neurotransmitter release. Thus, there is a need to develop minimal synthetic systems that have the neurotransmitter release characteristics observed in neurons and that allow manipulations and observations not possible in vivo.

Furthermore, such synthetic systems will set the stage for high-throughput screening of the effect of other factors on the process and could, ultimately, become a viable screening tool for the development of therapeutic leads to modulate neurotransmitter release and combat neurological disorders.

Previous attempts of in vitro reconstitutions of neurotransmitter release had fundamental limitations. Most of these systems did not directly probe the release of synaptic vesicle contents, but rather the exchange of lipids between vesicle and acceptor membranes. These experiments were based on the misconception that membrane lipid exchange (also referred to as "lipid mixing") is indicative of content release (also referred to as "content mixing"). Lipid mixing, which causes partial fusion between membranes (e.g., "hemifusion"), is necessary but not sufficient for pore formation between membranes and subsequent content release.

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The authors declare no conflict of interest.

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from synaptic vesicles. For example, lipid mixing occurs several seconds before content mixing in viral membrane fusion (5). There are other limitations to these earlier in vitro studies of neurotransmitter release: Often only a subset of the key constituents was used, and all previous assays utilized a constant 

Ca\(^{2+}\) concentration, rather than a stepwise increase. It has also become clear that synaptic vesicle fusion processes are inherently heterogeneous, so it is important to use single-particle and single-molecule techniques because the observation of the average of many such vesicles may obscure an accurate view of the underlying processes.

Here, we describe a synthetic single-vesicle system with reconstituted synaptic proteins that overcomes the limitations of the previous studies. Our system addresses the long-standing challenge to produce a millisecond content release burst upon Ca\(^{2+}\)-triggering, as monitored by a simultaneous content- and lipid-mixing assay with single vesicles. Prior to Ca\(^{2+}\) injection, the system is in a state of single interacting pairs of donor and acceptor vesicles, and fusion events are rare. Our system differentiates between single-vesicle interaction, membrane lipid exchange (e.g., hemifusion), and complete fusion (i.e., pore formation) upon Ca\(^{2+}\) injection, the latter mimicking quantized neurotransmitter release upon exocytosis of synaptic vesicles. Because single vesicles are observed and the time course of individual fusion events are monitored, our observations are analogous to quantized Ca\(^{2+}\)-triggered neurotransmitter release of one or more synaptic vesicles. We limited this study to neuronal SNARE, synaptotagmin, and complexin proteins. However, other synaptic proteins could be added to the system and their function examined.

An abbreviated overview of our experimental system is shown in Fig. P1A. Several molecules of synaptobrevin (a synaptic vesicle-associated SNARE protein) and synaptotagmin 1 (also a synaptic vesicle-associated protein) are reconstituted into “donor” vesicles, and several molecules of syntaxin (a SNARE protein that is primarily associated with the plasma membrane) are reconstituted into “acceptor” vesicles along with synaptosomal-associated protein 25A (SNAP-25), which forms a complex with syntaxin. Thus, the donor and acceptor vesicles are minimal mimics of synaptic vesicles and the target plasma membrane in the axonal terminal, respectively. Donor vesicles along with complexin molecules are introduced into a sample chamber with tethered acceptor vesicles. An incubation period follows, allowing the formation of a stable state of single interacting donor and acceptor vesicles (tethered to the surface) from which spontaneous fusion events are rare. A large excess of Ca\(^{2+}\)-containing solution is then rapidly injected into the sample chamber to achieve a defined Ca\(^{2+}\) concentration, and single-vesicle fusion events are observed with content- and lipid-mixing indicators using total internal reflection fluorescence microscopy.

Our synthetic system exhibits a rapid burst of complete fusion events upon Ca\(^{2+}\) injection (Fig. P1B). SNAREs, synaptotagmin 1, and complexin are all essential to obtain the most pronounced rapid burst of content mixing. The rapid burst of complete fusion events upon Ca\(^{2+}\) injection has a rise time (i.e., the characteristic time from zero fusion to maximum fusion) that is so fast that it cannot be resolved with our system. In analogy to the Ca\(^{2+}\) delivery method of our single-vesicle content-mixing system, in vivo experiments that employ photolysis of caged Ca\(^{2+}\) produce a stepwise increase of Ca\(^{2+}\) concentration in the axonal terminal. These in vivo experiments revealed a very rapid rise for presynaptic release and postsynaptic currents (approximately 1 ms), followed by a slower decay (<10 ms) at ambient temperature. It is noteworthy that the Ca\(^{2+}\)-triggered content release in our system shows a similar characteristic shape—i.e., a rapid rise followed by a slower decay.

The system used in our present study, most probably does not include all of the factors needed to reproduce the Ca\(^{2+}\) dose response and faster decay kinetics observed in vivo. For example, the proteins Munc18, RIM, Munc13, and Ca\(^{2+}\)-dependent activator protein for secretion (CAPS) play important roles in exocytosis, and their molecular function could be assessed with our system. It is remarkable, however, that our minimal system qualitatively mimics in vivo effects on Ca\(^{2+}\)-triggered fast synchronous release for key mutants of synaptotagmin 1 and complexin.

Our single-vesicle content-mixing system has shed more light onto the molecular mechanism of Ca\(^{2+}\)-triggered synaptic vesicle fusion (Fig. P1C). Neuronal SNAREs alone (i.e., without synaptotagmin and complexin) readily promote donor–acceptor vesicle interaction and some lipid mixing. Yet, neuronal SNAREs alone do not efficiently promote content mixing (i.e., complete fusion). The combination of SNAREs and synaptotagmin 1 effectively lowers the transition barriers to achieve complete fusion between membranes upon Ca\(^{2+}\) injection. We find that Ca\(^{2+}\) binding to synaptotagmin is essential for this process because mutation of one of the Ca\(^{2+}\) binding sites of synaptotagmin 1 greatly reduces the rapid fusion burst upon Ca\(^{2+}\) injection. Complexin significantly increases the rapid fusion burst at higher Ca\(^{2+}\) concentrations. However, complexin is not absolutely required to observe Ca\(^{2+}\)-triggered fusion; SNAREs and synaptotagmin are sufficient and constitute a most minimal Ca\(^{2+}\)-triggered fusion system, albeit less efficiently than in the presence of complexin. This conclusion was not achieved in previous studies that only used lipid-mixing indicators. Such work concluded that neuronal SNAREs alone produce significant lipid-mixing activity. Because lipid mixing was misinterpreted at the time as complete fusion, it was thought that some sort of fusion clamp must always exist to prevent full fusion in the absence of Ca\(^{2+}\). Our results now show that such a clamp is not absolutely required for neuronal SNAREs: Little complete fusion is observed in the starting state of interacting vesicles until Ca\(^{2+}\) arrives, even in the absence of complexin.

The discoveries presented in this paper demonstrate the power of developing an in vitro assay that differentiates the different stages leading to full vesicle fusion, and how this assay allows the exploration of the role of different molecular components in vesicle fusion. Our single-vesicle system with simultaneous lipid- and content-mixing monitoring provides a platform to study the role of the various factors in Ca\(^{2+}\)-triggered synaptic vesicle fusion (in addition to the minimal set of proteins used in this study). This work illustrates the advantages of the synthetic system: One can deliberately omit or manipulate components to determine which parts are essential and monitor what happens to the system when essential parts are missing. By directly observing individual protein–protein interactions in conjunction with single-vesicle content and lipid-mixing indicators, our assay will help provide a foundation to investigate the molecular mechanism of synaptic neurotransmitter release.

Inside insight to membrane fusion

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Exocytosis of synaptic vesicles is a central step in neuronal signal transmission (reviewed in 1). On arrival of an action potential, voltage gated Ca\(^{2+}\) channels in the synaptic plasma membrane open. The influx of Ca\(^{2+}\) ions triggers fusion of synaptic vesicles with a delay of less than 1 ms and increases the fusion rate by more than four orders of magnitude (2). During the past 2 decades, major progress has been made in deciphering the molecular events underlying exocytosis of synaptic vesicles. Although details are still controversial, it is widely accepted that membrane fusion itself is catalyzed by the synaptic SNARE proteins, including the R-SNARE synaptobrevin/vesicle-associated membrane protein that resides on synaptic vesicles and the Q-SNAREs syntaxin-1 and synaptosomal-associated protein-25 (SNAP-25) that reside in the presynaptic plasma membrane. On contact, these SNAREs form a helical complex that is initiated at the membrane-distal N-terminal ends and progresses toward the C-terminal transmembrane anchors, thus pulling the membranes together. The tight coupling of fusion to Ca\(^{2+}\) influx is mediated by the protein synaptotagmin, a resident of synaptic vesicles. Synaptotagmin-1 bears two Ca\(^{2+}\)-binding C2 domains that interact with both SNAREs and acidic membrane lipids in a Ca\(^{2+}\)-dependent manner. However, despite intense research by many groups, it is still unclear exactly how the enormous acceleration by Ca\(^{2+}\) is achieved at the molecular level. Kyoung et al. (3) now provide us with a unique assay in which some of the key steps have been reconstructed in vitro, thus opening the door toward unraveling synaptic membrane fusion.

Reconstitution of a complex cellular event in vitro, such as protein-mediated membrane fusion, is an important step toward a full mechanistic understanding, and despite the increasing sophistication of cell-based assays, there is still no alternative to this approach. Indeed, there is a long tradition in reconstituting fusion events using both biological and artificial membranes; such assays have been instrumental in unraveling the mechanisms underlying membrane merger, particularly in the field of viral fusion proteins (4). Similarly, the presence of appropriate combinations of SNAREs suffices to mediate fusion of artificial membranes: Liposomes containing synaptobrevin-2 are capable of lipid mixing with a second population of liposomes that contain SNAP-25 and syntaxin-1A (5). Although this observation has been reproduced numerous times, major problems persist. First, the fusion rate is extremely slow (at least 5 orders of magnitude slower than neuronal exocytosis). Second, including the Ca\(^{2+}\) sensor synaptotagmin-1 in the experiments only results in a very moderate acceleration of fusion (reviewed in 6 and 7) that is by no means even close to the increase of four orders of magnitude observed in neurons (2).

A major limitation of these experiments is the “bulk” nature of the measurements. In typical fusion assays, liposomes are labeled with two spectrally separated fluorescent lipid analogs. These lipid analogs mix on membrane fusion, resulting in a change of fluorescence attributable to Förster resonance energy transfer (FRET) (5). This process is usually measured in a cuvette, where total fluorescence is measured over time after mixing of the two populations. Although this assay is simple and very reliable, it is not possible to distinguish docking from fusion with this approach. This is a serious limitation because the first membrane tethering step (Fig. 1; step 1) is rate-limiting for native SNAREs (8, 9). Thus, any effect on fusion downstream of tethering, as is proposed for synaptotagmin-1 (6), cannot be reliably measured. Furthermore, ensemble averaging does not allow for distinguishing between vesicle populations exhibiting different kinetics, and multiple rounds of fusion cannot be differentiated. Last but not least, testing for nonleakiness (an important criterion for biological fusion) requires different dyes, is difficult to carry out in the same experiment, and is thus frequently omitted.

In single-liposome fusion assays, membrane tethering can be easily discerned from actual lipid mixing and multiple rounds of membrane fusion can also be readily observed. The first researchers to realize single vesicle Ca\(^{2+}\)-triggered membrane fusion experimentally were Lee et al. (10). In this study, Q-SNARE liposomes were immobilized via biotin-avidin linkage (11) on a glass surface. This allowed for measuring lipid mixing with R-SNARE liposomes containing the Ca\(^{2+}\) sensor synaptotagmin-1 using fluorescence microscopy. Surprisingly, membrane fusion was accelerated already at 10 μM Ca\(^{2+}\) (10), which is far lower than observed previously in vitro. Even more surprisingly, higher concentrations resulted in a decrease of the fusion efficiency. This paradox was attributed to “back binding” by synaptotagmin to its “own” membrane at higher Ca\(^{2+}\) concentrations, resulting in functional inactivation (12). Notwithstanding these important findings and methodological advances, content leakage and hemi/full-membrane fusion were not addressed in this study.

Kyoung et al. (3) have now overcome these limitations in a very elegant manner. As in the study by Lee et al. (10), Q-SNARE liposomes were immobilized on a glass slide and synaptotagmin/R-SNARE liposomes were subsequently introduced. Membrane fusion was then monitored by a cunning triple-fluorescence microscopy approach. First, Ca\(^{2+}\) was introduced together with the fluorescent dye cascade blue, which allowed for monitoring the Ca\(^{2+}\) diffusion through the microscopy chamber. Second, one of the liposome populations contained a self-quenching concentration of the red fluorescent lipid analog 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine 4-chlorobenzensulfonate. Thus, lipid mixing could be monitored because dilution of the lipids resulted in decreased self-quenching and an increase in fluorescence. Third, a self-quenching concentration of the green fluorophore sulforhodamine-B was encapsulated in the lumen of one of the liposome populations. Leakage and content mixing could hence be directly followed by monitoring dispersion and/or increase of the fluorescence. Because these three fluorophores are spectrally separated, Ca\(^{2+}\) diffusion, as well as membrane tethering and lipid and content mixing, could be followed simultaneously.
The results contain a few surprises. First, SNAREs alone mediated some tethering but hardly sufficed to catalyze membrane fusion: Only 20% of the tethered liposomes fused within ~30 min. Tethering was enhanced in the presence of complexin [as reported previously (9, 13)] or with synaptotagmin (7, 14). In the presence of synaptotagmin-1, Ca\(^{2+}\) dramatically increased fusion. Fusion consisted of a fast phase with a time constant well below the 200-ms time resolution of other regulatory proteins, such as Munc18, Munc13, and Rab3-interacting molecule (1, 6). Also, with direct evidence that trans-SNARE complex formation can, in fact, mediate tethering, it will be very interesting to determine how far the SNARE motifs are coiled up and how fast further zippering is arrested. Particularly attractive is the perspective of combining the assay of Kyoung et al. (3) with an earlier single-molecule FRET approach from the same group (15).

The study by Kyoung et al. (3) introduces a unique tool for studying membrane fusion. Most importantly, or perhaps even worryingly, they show profoundly different kinetics of lipid and content mixing, asking for a reevaluation of the many studies on membrane fusion that only looked at lipid mixing. The triple-fluorescence single-liposome fusion assay may allow resolution of some of the questions that have been occupying the SNARE field for many years.

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