Two Distinct Modes of Exocytotic Fusion Pore Expansion in Large Astrocytic Vesicles*§

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Background: Astrocytes release glutamate and D-serine by fusion of large vesicles.

Results: Fusion occurs at a ring shape of the docked membrane, and the movement of fusion-produced membrane fragment expands the fusion pore.

Conclusion: Two modes of fusion pore expansion are associated with full and partial collapses of large vesicles.

Significance: Two fusion modes provide a novel mechanism for full and partial collapses of large secretory vesicles.

Formation of the fusion pore is a central question for regulated exocytosis by which secretory cells release neurotransmitters or hormones. Here, by dynamically monitoring exocytosis of large vesicles (2–7 μm) in astrocytes with two-photon microscopy, we found that the exocytotic fusion pore was generated from the SNARE-dependent fusion at a ring shape of the docked plasma-vesicular membrane and the movement of a fusion-produced membrane fragment. We observed two modes of fragment movements, 1) a shift fragment that shifted to expand the fusion pore and 2) a fall-in fragment that fell into the collapsed vesicle to expand the fusion pore. Shift and fall-in modes are associated with full and partial collapses of large vesicles, respectively. The astrocytic marker, sulforhodamine 101, stained the fusion-produced membrane fragment more brightly than FM 1-43. Sulforhodamine 101 imaging showed that double stained the fusion-produced membrane fragment more brightly than FM 1-43. Sulforhodamine 101 imaging showed that double fusion pores could simultaneously occur in a single vesicle (16% of large vesicles) to accelerate discharge of vesicular contents. Electron microscopy of large astrocytic vesicles showed shift and fall-in membrane fragments. Two modes of fusion pore formation demonstrate a novel mechanism underlying fusion pore expansion and provide a new explanation for full and partial collapses of large secretory vesicles.

Neurotransmitters and hormones are released by neuronal terminals and secretory cells through the regulated exocytosis of vesicles. The exocytosis process starts with the docking of a vesicle to the cell membrane promoted by soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complexes (1–3). The SNAREs then lead to fusion between the vesicular membrane and the plasma membrane, forming the fusion pore for discharge of vesicular contents (4). The initial fusion pore is a nanometer-sized channel through which neurotransmitter molecules can only diffuse slowly out of the vesicle (5–7). The fusion pore is then expanded, leading to a massive release of neurotransmitters (8, 9). Although amperometric foot signals have been observed during expansion of the fusion pore (6, 10–13), the detailed mechanism underlying fusion pore expansion is unknown.

Although there is a debate about the role of astrocytic Ca2+ signaling and Ca2+-dependent release in synaptic plasticity (14–16), astrocytic release of glutamate was broadly reported to modulate both excitatory (17–22) and inhibitory synaptic transmission (23, 24) under physiological or pathological conditions such as epileptic seizures (25–27). One type of astrocytic glutamate release that activates a slowly decaying transient inward current (SIC)3 in neurons (28–31) is through exocytotic fusion of a large astrocytic vesicle in a SNARE-dependent manner (25, 32). Large astrocytic vesicles also contain D-serine (33), and co-release of glutamate and D-serine induces NMDA receptor-mediated neuronal SICs. The size of these large astrocytic vesicles (∼3 μm) is similar to vacuoles in yeast (34) and about 100 times larger than synaptic vesicles (∼30 nm). Fusion at a ring-shaped membrane microdomain surrounding the apposed boundary membrane has been found in intracellular homotypic fusion between yeast vacuoles (34–36). Here, we report a similar mechanism underlying exocytotic fusion in large astrocytic vesicles. We directly observed the formation of the fusion pore in large vesicles by fluorescence imaging under two-photon microscopy. We further demonstrate that two distinct modes of fusion pore expansion, shift mode and fall-in mode, are associated with full and partial collapses of large astrocytic vesicles.

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The abbreviations used are: SIC, slowly decaying transient inward current; ACSF, artificial cerebrospinal fluid; DIC, differential interference contrast; AO, acridine orange; LC-TT, light-chain tetanus toxin; GFAP, glial fibrillary acid protein; Sb2, synaptobrevin 2.
**EXPERIMENTAL PROCEDURES**

A total of thirty 15–30-day-old (postnatal days 15–30) Sprague-Dawley rats were used. All experiments were approved by the Institutional Animal Care and Use Committee at the New York Medical College following the National Institutes of Health guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

**Slice Preparation**—Brain slices were prepared as described previously (23). Briefly, Sprague-Dawley rats of either sex were deeply anesthetized with sodium pentobarbital (55 mg/kg) and then decapitated. Transverse brain slices of 300–μm thickness were cut with a vibratome (Technical Products International, St. Louis, MO) in a cutting solution containing (in mM): 2.5 KCl, 1.25 NaH2PO4, 10 MgSO4, 0.5 CaCl2, 10 glucose, 26 NaHCO3, and 230 sucrose. Slices containing the hippocampus were incubated in artificial cerebrospinal fluid (ACSF) gassed with 5% CO2, 95% O2 for 1–4 h and then transferred to a recording chamber (1.5 ml) perfused continually (3 ml/min) with ACSF for recording and imaging. ACSF contained (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH2PO4, 2 MgCl2, 2 CaCl2, 10 glucose, and 26 NaHCO3 (pH at 7.4 when gassed with 5% CO2, 95% O2).

**Patch Clamp Recording**—CA1 pyramidal cells in hippocampal slices were visualized with a 60×/0.90 water immersion lens on an Olympus BX51 upright microscope (Olympus Optical Co.) equipped with infrared differential interference contrast (IR-DIC) optics. Patch pipette electrodes (5–10 megaohms) were pulled from KG-33 glass capillaries (inner diameter 1.0 mm, Garner Glass Co., Claremont, CA) using a P-97 electrode puller (Sutter Instrument Co., Novato, CA). The patch pipette solution contained (in mM): 123 potassium gluconate, 10 KCl, 1 MgCl2, 10 HEPES, 1 ATP, 0.2 GTP, and 4 glucose (pH adjusted to 7.2 with KOH). Recorded signals were filtered through an eight-pole Bessel low pass filter with a 2-kHz cut-off frequency and sampled using the PCLAMP 10.2 gated second antibody (goat anti-rabbit IgG, 1:250 by 1% donkey serum, 0.3% Triton X-100, 0.1% Triton X-100, PBS, Millipore, Billerica, MA) for 24 h followed by a fluorescein isothiocyanate (FITC)-conjugated second antibody (goat anti-rabbit IgG, 1:250 by 1% donkey serum, 0.1% Triton X-100, PBS, Millipore) for 2 h at room temperature. Finally, antibody fluorescence in the SR101-labeled area was examined under two-photon microscopy.

**RESULTS**

**Exocytotic Fusion of Large Astrocytic Vesicles**—Our previous studies have demonstrated that astrocytes release glutamate and d-serine through fusion of large vesicles (25, 32, 33). To monitor the fusion process of large astrocytic vesicles, we puffed glutamate (50 μM) together with FM 1-43 (20 μM) in the presence of tetrodotoxin (1 μM) and imaged FM 1-43 fluorescence using two-photon microscopy. FM 1-43 has a hydrophilic head with two positive charges that limit its translocation through the bilayer membrane and a four carbon hydrophobic tail that has a high affinity for hydrophobic lipids (37, 38). These characteristics allow FM 1-43 to bind to the fused (broken) membrane, and only occasionally and reversibly bind to the intact membrane. A single puff induced very few large vesicles (supplemental Fig. S1A, Puff 1), and FM 1-43 fluorescence in the extracellular space quickly declined after each puff. Repet-
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FIGURE 1. Shift mode of fusion. A, a representative large vesicle (v) was collapsing with a fragment of membrane (arrowheads) that shifted intravesicularly. Scale bar, 5 μm. B, left traces, changes in FM 1-43 fluorescence in the membrane fragment (red, measured in the circled area in A) and the intravesicular lumen (black) of the vesicle in A). Pun, puncta. Right bar graph, the mean increase in FM 1-43 fluorescence (ΔF/Fo) in the shift membrane fragment (red bar) and the intravesicular lumen of collapsed vesicles (black bar). The number in the red bar indicates the sample number of vesicles. *** denotes p < 0.001 as compared with the lumen, Student’s paired t test. C, FM 1-43 fluorescence (ΔF/Fo) in the shift puncta (red circles) or the membrane of uncollapsed vesicles (open circles) was plotted against time (0 s, the start of vesicular collapse). n = 20 vesicles. All averaged data are presented as mean ± S.E.

Positive puffs induced formation of more large vesicles around the puffer pipette (supplemental Fig. S1A, Puff 3 and Puff 6, v). An FM 1-43-negative large vesicle was identified as a newly appearing round structure with low fluorescence surrounded by higher FM 1-43 fluorescence. These large vesicles could also be detected by DIC microscopy (supplemental Fig. S2, A–C), by patch clamp loading of astrocytes with Alexa Fluor®-594 and Fluo-4 potassium, or by Fluo-4 AM loading of astrocytes (25, 32, 33). Repeated puffs increased FM 1-43 fluorescence around large vesicles, probably due to increased endocytosis of FM 1-43 (39). After 3–5 puffs (6–10-min imaging), FM 1-43-stained small puncta with high fluorescence intensity appeared (supplemental Fig. S1B, arrow). The appearance of FM 1-43 puncta was accompanied by the collapse of large vesicles (supplemental Fig. S1B, i–iv and v), suggesting that these puncta are associated with the fusion. As a vesicle fused with the cytoplasm membrane, FM 1-43 flowed into the vesicle through the fusion pore and bound to the fused membrane near the fusion pore, forming a high fluorescence intensity punctum. These large vesicles continued to fuse after puffs were stopped. Whole-cell recording in CA1 pyramidal neurons whose apical dendrites passed through the imaging area showed a SIC immediately followed by the start of vesicular collapse (supplemental Fig. S1C, SIC), indicating that the fusion caused glutamate release. Large astrocytic vesicles were also induced by puffing ACSF (weak mechanical stimulation) in the tetrodotoxin. The fusion of puffing ACSF-induced large vesicles resulted in D-serine-mediated enhancement of NMDA receptor activation without SICs (33).

Two Modes of Fusion Pore Formation—We observed two types of fusion events in large vesicles, shift mode and fall-in mode. A representative large vesicle with the shift mode fusion is shown in Fig. 1A (supplemental Movie 1). When the large vesicle started to collapse, the fluorescence intensity in a small area of the vesicular membrane transiently decreased (Fig. 1A, 0–6 s, arrow, and 1B, red trace/arrow), indicating that a fusion pore was formed in this area. Then, a piece of membrane near the fusion pore gradually became stained with FM 1-43 (Fig. 1A, 12–60 s, arrow, and 1B, red trace and bar). In contrast, the intravesicular lumen remained unstained (Fig. 1A, v, and 1B, black trace and bar). The FM 1-43 fluorescence intensity in the shift membrane fragment gradually increased following vesicular collapse (Fig. 1C, red circles), indicating that FM 1-43 dye continued to bind to the shift membrane fragment and that the fusion event was exocytotic, rather than intracellular homotypic fusion. The collapsed vesicle did not fuse into the adjacent vesicle (Fig. 1A, v), further supporting exocytotic fusion. The fluorescence intensity in the intact membrane of uncollapsed vesicles remained unchanged (Fig. 1C, open circles), suggesting that the increase in the fluorescence intensity in the punctum was not due to phototoxicity and embedding of FM 1-43 into the intact membrane. Puffing glutamate-induced large vesicles and their collapses could be detected by DIC microscopy without any dyes (supplemental Fig. S2, A and B), indicating that large...
vesicles and fusion events were not due to phototoxicity and dye embedding into the cytoplasmic and vesicular membrane.

In the fall-in mode, a membrane fragment fell into the vesicle when it started to collapse (Fig. 2A, arrowhead) and then moved in Brownian motion in the collapsed vesicle (supplemental Movie 2). The fall-in membrane fragment was also gradually and increasingly stained by FM 1-43 following collapse (Fig. 2, A and B, red circles), whereas fluorescence in the intact membrane of uncollapsed vesicles was unchanged (Fig. 2B, open circles). The fall-in mode of fusion and partial vesicular collapse were observed under DIC microscopy (supplemental Fig. S2B), confirming that this fusion mode is not due to phototoxicity and dye embedding into the membrane. The majority of large vesicles (66%, 78/118) collapsed with the shift membrane fragment (Fig. 2C, Shift), whereas 30% (35/118) collapsed with the fall-in membrane fragment (Fig. 2C, Fall-in). Both the shift and the fall-in fragments preferentially moved into, rather than out of, collapsed vesicles during fusion pore expansion, suggesting that the direction of fragment movement is controlled by intravesicular mechanisms. Only 5 out of 118 vesicles (Fig. 2C, None) collapsed without membrane fragments, which might have fallen either out into the extracellular space or out of focus. No vesicles contained the fall-in membrane fragment after three or fewer puffs (Fig. 2D, Puff 3), whereas 22.7 ± 1.6% of large vesicles (108/469 imaged vesicles) contained the fall-in membrane fragment following 10 puffs (Fig. 2D, Puff 10), indicating that the fall-in mode requires stronger stimulation than the shift mode.

Partial and Full Collapses in Two Modes—Partial collapse was more frequently observed in the fall-in mode (Fig. 2A, 48 s) as compared with the shift mode (Fig. 1A). We further analyzed changes in vesicular volume in the two fusion modes. Vesicular volume was calculated using vesicular diameter measured by X-Y-Z-T scanning. The mean vesicular volume before fusion was 41.4 ± 8.1 μm³. Relative changes in each vesicle volume (V/VO) during fusion showed that partial collapse of large vesicles in the fall-in mode produced a smaller volume reduction (Fig. 3A, □) than the shift mode (Fig. 3A, ●, p < 0.001, two-way analysis of variance). Almost one-half of fusion events with the fall-in mode (46%, 16/35) showed less than a 50% volume reduction (Fig. 3B, inset, Fall-in), whereas none did in the shift mode (Fig. 3B, inset, Shift). The volume reduction rate (ΔV/Δt) showed that collapse stopped earlier in the fall-in mode (Fig. 3B, ●) than in the shift mode (Fig. 3B, ○), suggesting that the exocytotic fusion pore closes earlier in the fall-in mode than in the shift mode.

SR101 Stains Puncta More Brightly—SR101, activity-dependent dye (40–42) and astrocyte-selective fluorescence marker (43), has been used in marking astrocytes, detecting exocytosis (42), and bleaching FM 1-43 dye in synaptic vesicles with “kiss and run” fusion mode (44, 45). To further examine fusion pore formation in large astrocytic vesicles, we used SR101 to monitor the collapse of large astrocytic vesicles. SR101 (1 μM) was applied in the perfusion solution for 10 min and then washed out for 10 min to reduce the background fluorescence. In this experiment, we used weak mechanical stimulation (puffing...
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ACSF) to induce large vesicles (33). Three to five puffs induced multiple SR101-negative large vesicles (supplemental Movie 3). The collapse of large vesicles was accompanied by a bright fluorescence punctum (Fig. 4, A and B, arrowheads). The majority of large vesicles (80%, 78/98) collapsed with a single punctum (Fig. 4A, arrowheads, and supplemental Movie 4). A subgroup of large vesicles (16%, 16/98) collapsed with two puncta (Fig. 4B, arrowheads, and supplemental Movie 5), indicating that two fusion pores were formed simultaneously. Only a few large vesicles (4%, 4/98) collapsed without puncta. SR101 fluorescence puncta also preferentially moved into, rather than out of, collapsed vesicles. The collapse speed of large vesicles measured by SR101 and FM 1-43 was similar (Fig. 4C, ○ versus ●).

The SR101 fluorescence intensity in the puncta increased with time (Fig. 5A, ○, 20–60 s, p < 0.001 as compared with 0 s, Student’s paired t test, n = 15 vesicles for each group), indicating that SR101 entered the collapsed vesicles. The SR101 fluorescence intensity in the puncta was 2.8 ± 0.2 times that in the nearby tissue (p < 0.001, Student’s paired t test, n = 15 vesicles for each group), suggesting that SR101 binds to the fused membrane with a higher affinity than to the intact cytoplasmic and vesicular membrane. The SR101 fluorescence intensity in the puncta (Fig. 5A, ○) was significantly higher than FM 1-43 in the puncta (Fig. 5A, ●, p < 0.001, two-way analysis of variance, n = 15 vesicles for each group). The time for t50 (∆F reaching at 50% of ∆Fmax) of SR101 (Fig. 5B, ○, t50 = 4.8 ± 1.1 s) was significantly shorter than that of FM 1-43 (Fig. 5B, ●, t50 = 18.0 ± 6.8 s, p < 0.001, Student’s unpaired t test, n = 15 vesicles for each group). These results suggest that SR101 binds to the membrane fragment with a higher affinity than FM 1-43. In contrast to low FM 1-43 fluorescence in the vesicular lumen (Fig. 5C, ●), SR101 fluorescence in the vesicular lumen also increased significantly (Fig. 5C, ○, 10–25 s, p < 0.001 as compared with 0 s, Student’s paired t test, n = 15 vesicles for each group). To further compare fluorescence increases in vesicular puncta and lumen, we calculated the ratio of punctum fluorescence increases versus lumen fluorescence increases (∆Fpun/∆Flum). The ratio (∆Fpun/∆Flum) for FM 1-43 was 19.0 ± 3.1 (Fig. 5D, ●), whereas for SR101, it was only 1.8 ± 0.3 (Fig. 5D, ○), suggesting that FM 1-43 only binds to the fused membrane fragment, whereas SR101 binds to both the fused membrane fragment and the intact internal membrane.

Ultrastructures of Membrane Fragments—To confirm the observation of FM 1-43 or SR101-stained membrane fragments, we performed transmission electron microscopy (EM) to examine large vesicles in astrocytes from glutamate-treated versus untreated slices. Astrocytes were labeled with an anti-GFAP antibody. The EM micrograph in Fig. 6A showed multiple large vesicles around the soma of the GFAP-labeled soma of an astrocyte (Fig. 6A, left, Ast). Large vesicles were identified by their round shape with single bilayer membranes and almost plain internal contents. Fall-in membrane fragments (Fig. 6A, right, solid arrows) were identified by isolated membrane fragments in collapsed large vesicles (Fig. 6A, right, Vc), and shift membrane fragments (Fig. 6, A and B, hollow arrows) were identified by a round and dark structure that was associated with the membrane of collapsed large vesicles (Fig. 6, A and B, Vc). Membrane fragments were observed in 67% (263/391) of large vesicles. The size of large vesicles with membrane fragments (Fig. 6D, Vc) was significantly smaller than large vesicles without fragments (Fig. 6D, V, p < 0.001, Student’s unpaired t test), indicating that fusion and collapse had occurred in the fragment-containing vesicles. In control untreated slices (Fig. 6C), no large vesicles with membrane fragments were found. More than one-half of membrane fragments (58%, 164/283) were connected with a filament (Fig. 6, A and B, arrowheads), suggesting that the filament might regulate intravesicular direction of fragment movement. The filament connecting the membrane fragment is likely composed of actins (46–48). The mean diameter of the fall-in membrane fragments measured by EM images (Fig. 6E, solid bar, EM, 0.2–1.4 μm) was not significantly different from the diameter of the FM 1-43 puncta (Fig. 6E, solid bar, FM, 0.3–1.5 μm, p = 0.40, unpaired t test). In contrast, the mean size of the shift FM 1-43 or SR101 puncta (Fig. 6E, open bar, FM or SR, 0.5–1.8 μm) was significantly larger than the EM shift membrane fragments (Fig. 6E, open bar, EM, 0.3–0.7 μm, p < 0.001, Student’s unpaired t test). The explanation for these results is that, in the fall-in mode, the FM 1-43 punctum only contains FM 1-43 in the fall-in fragment (see...
SNARE Dependence of Large Vesicle Fusion — To test whether the fusion of large astrocytic vesicles depends upon SNAREs, we first used light-chain tetanus toxin (LC-TT) that enzymatically cleaves the SNARE protein synaptobrevin to block fusion of large vesicles. Astrocytes were patched with the pipette solution containing 50 mM glutamate and 2 μM inositol 1,4,5-trisphosphate that stimulated astrocytes to generate large vesicles (25). Acridine orange (AO, 1 μM) that stains acidic organelles and tetrodotoxin (1 μM) were added to the ACSF slice solution to image large astrocytic vesicles. AO imaging showed that multiple large vesicles in patched astrocytes were AO-negative (Fig. 7A), indicating that large vesicles are not acidic organelles. In the absence of LC-TT, the collapse of AO-negative large vesicles was observed frequently (Fig. 7A, arrowheads, and supplemental Movie 6). When LC-TT (0.2 μM) was added to the astrocyte patch pipette, the vesicular collapse was blocked (Fig. 7B, and supplemental Movie 7). LC-TT reduced the fusion rate significantly (Fig. 7D, AO, LC-TT versus Con, p < 0.01, Student’s unpaired t test). The SNARE dependence of large vesicle fusion was further supported by the LC-TT blockade of the disappearance of Alexa Fluor®-594/Fluo-4-positive large vesicles (Fig. 7, C and D, Alexa/Fluo-4), similarly as described in our previous studies (32, 33).

To confirm the SNARE dependence of large vesicle fusion, we used the antibody against Sb2, a component of the SNARE complex, which was reported to express in astrocytes (49, 50). Glutamate (50 mM) and SR101 (10 μM) were repetitively puffed (10 times) into the CA1 stratum radiatum to induce large vesicles. SR101 was able to label the puffed area and puncta. Immunofluorescence imaging of anti-Sb2 in the puffed area with high SR101 fluorescence intensity showed that the majority of SR101 puncta (Fig. 7E, SR101, arrow, and 7G, Puff, SR) were
The unpuffed area with the low SR101 fluorescence intensity in the same slice was used as a control. In the control area, very few puncta and low levels of anti-Sb2 were found (Fig. 7, F–G, Con). The number of anti-Sb2/SR101-labeled puncta in the puffed area (Fig. 7G, Puff, Sb2) was significantly larger than in the unpuffed area (Fig. 7G, Con, Sb2, p < 0.001, Student’s unpaired t test). These results suggest that high levels of the Sb2 protein are present in the fusion-produced membrane fragment, and the number of Sb2 proteins in the docking area (Fig. 8, Docking, blue circle) increases when a large vesicle docks with the cytoplasmic membrane.

**DISCUSSION**

Our previous studies have suggested that astrocytic glutamate release by large vesicles is SNARE-dependent (25, 32, 33). Here, we demonstrate that the fusion occurs at a ring shape of the docked membrane microdomain, and the movement of the membrane fragment into vesicles underlies expansion of the exocytotic fusion pore. Occurrence of homotypic fusion at a “vertex” ring around the disc of apposed boundary in yeast vacuoles has been reported (34–36, 46). Our study demonstrates that fusion at the ring shape of the docked membrane is also used to form the exocytotic fusion pore in large astrocytic vesicles.

Based on our results, we propose the following model for two modes of exocytotic fusion pore formation. First, a ring shape of docked vesicular membrane is bridged to the plasma membrane by the SNARE complex (Fig. 8, Docking, Vertical view, blue ring). In the fall-in mode, the SNARE complex bridges the full ring of vesicle-plasma membranes (Fig. 8, Fall-in mode, Docking, Vertical view, blue ring), whereas in the shift mode, a small piece of membrane in the ring is not bridged (Fig. 8, Shift mode, Docking, Vertical view, arrowhead). Second, fusion between vesicular and plasma membrane occurs at the ring to form membrane fragments (Fig. 8, Fusion). In the fall-in mode, the whole ring is fused (Fig. 8, Fall-in mode, Fusion), whereas in the shift mode, a small piece of the ring is not fused (Fig. 8, Shift mode, Fusion, Vertical view, arrowhead). Third, the membrane fragment moves intravesicularly due to the action of a connected filament (Fig. 8, Expansion, F). In the fall-in mode, the membrane fragment detaches from the vesicular membrane and falls into the collapsed vesicle to expand the fusion pore (Fig. 8, Fall-in mode, Expansion). In the shift mode, the membrane fragment shifts to the side to expand the fusion pore (Fig. 8, Shift mode, Expansion). The fall-in mode often results in partial collapse, probably due to resealing of the fusion pore (Fig. 8, Fall-in mode, Collapse). In our experiments, we observed that a partially collapsed vesicle could enlarge and fuse again. The shift mode mostly results in full collapse of the vesicle (Fig. 8, Shift mode, Collapse). Why the fusion pore with shift fragments usually resulted in full collapse, whereas fall-in fragments did not, is unknown. One possible explanation is that...
the connection between the shift membrane fragment and the vesicular membrane physically blocks resealing of the fusion pore (Fig. 8, Shift mode, Collapse).

The homotypic fusion between two large vesicles occasionally occurred without the appearance of FM 1-43 puncta (supplemental Movie 8). We believe that shift or fall-in mode fusion events observed with FM 1-43 or SR101 were exocytotic, rather than intracellular homotypic fusion, between vesicles, because: 1) homotypic fusion is accompanied by an enlarging vesicle and a disappearing vesicle, whereas fusion with shift or fall-in fragment was associated with only one collapsed vesicle; 2) extracellularly applied FM 1-43 stained the shift or fall-in membrane fragment, whereas in homotypic fusion, membrane fragments were not stained (supplemental Movie 8); 3) the FM 1-43 fluorescence intensity in the shift or fall-in membrane fragments increased with time, suggesting that extracellular FM 1-43 dye is flowing into the collapsed vesicle and binding to the membrane fragment; and 4) the coincidence of vesicular collapses with SICs confirms their exocytotic fusion and release of glutamate into the extracellular space. It should be noted that in EM experiments, a portion of fusion-produced membrane fragments could have resulted from intracellular homotypic fusion because homotypic fusion could also produce intravesicular fragments (34).

The different fusion modes could not be due to the changed fluidity resulting from the dye embedding into the lipid bilayer because: 1) all fusion events were blocked by light chain of tetanus toxin (Fig. 7, Shift mode, Collapse); and 2) anti-Sb2 antibody stains the fusion-produced membrane fragment (Fig. 7, E), confirming the SNARE dependence of the fusion; 3) the fall-in mode of fusion was observed under DIC microscopy without any dyes (supplemental Fig. S2B); 4) both full and partial collapses of large vesicles could be detected by the membrane-permeable fluorescence dye, acridine orange (supplemental Movie 9); and 5) EM results showed two modes of membrane fragments (Fig. 6). The partial collapse of large vesicles with the fall-in mode could re-enlarge and refill glutamate release mechanisms (supplemental Movie 10) and then undergo the second release, whereas the full collapse of large vesicles with the shift mode never re-enlarged and re-collapsed. Therefore, a single large vesicle uses the fall-in mode to release glutamatergic transmission repetitively. Because the fall-in mode of fusion only occurred after a large number of large vesicles were generated and fused, cells may use this fusion mode to reduce energy consumption.

Whether fusion at the ring shape of the docked membrane is a universal mechanism underlying formation of all exocytotic fusion pores, including neurotransmitter release from axonal terminals, or just used in fusion of large secretory vesicles, is unknown. However, results demonstrating that FM 1-43 fluorescence in the vesicular lumen did not increase during vesicular collapse (Fig. 1B, black) suggest a low affinity of FM 1-43 to
the intact intravesicular membrane. Therefore, the FM 1-43 fluorescence staining of synaptic vesicles after vesicle recycling may result from FM 1-43 binding of the fused membrane rather than the intact intravesicular membrane.

Higher binding affinity of SR101 than FM 1-43 suggests that SR101 is a better fluorescence marker for detecting the fusion pore than FM dyes. By imaging SR101 fluorescence, we found for the first time that multiple fusion pores could be formed during exocytosis of a large secretory vesicle to accelerate discharge of vesicular contents. The mechanism that determines the synchronization of multiple fusion pores is unknown and requires further studies.

Fusion at the ring shape of the docked membrane followed by the movement of the membrane fragment demonstrates a novel mechanism for expansion of the exocytotic fusion pore in large vesicles. Two distinct modes of fusion expansion, shift mode and fall-in mode, provide a new explanation for full and partial collapses of large secretory vesicles.

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