Post-fusion structural changes and their roles in exocytosis and endocytosis of dense-core vesicles

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Vesicle fusion with the plasma membrane generates an Ω-shaped membrane profile. Its pore is thought to dilate until flattening (full-collapse), followed by classical endocytosis to retrieve vesicles. Alternatively, the pore may close (kiss-and-run), but the triggering mechanisms and its endocytic roles remain poorly understood. Here, using confocal and stimulated emission depletion microscopy imaging of dense-core vesicles, we find that fusion-generated Ω-profiles may enlarge or shrink while maintaining vesicular membrane proteins. Closure of fusion-generated Ω-profiles, which produces various sizes of vesicles, is the dominant mechanism mediating rapid and slow endocytosis within ~1–30 s. Strong calcium influx triggers dynamin-mediated closure. Weak calcium influx does not promote closure, but facilitates the merging of Ω-profiles with the plasma membrane via shrinking rather than full-collapse. These results establish a model, termed Ω-exo-endocytosis, in which the fusion-generated Ω-profile may shrink to merge with the plasma membrane, change in size or change in size then close in response to calcium, which is the main mechanism to retrieve dense-core vesicles.
Results

Imaging Ω-profile at the instant of fusion. To label Ω-shaped membrane profiles and assess its structural changes, primary cultured bovine chromaffin cells were bathed with membrane-impermeable fluorescent dyes (Fig. 1a)25. With Alexa Fluor 647 (A647, 30 μM) in the bath, cells were voltage clamped while imaged confocally every 5–15 ms at the cell-bottom (applies if not mentioned otherwise, Fig. 1a). A 1 s depolarization (depol1s, from −80 mV to +10 mV, if not mentioned otherwise) induced a calcium current (ICa) of 309 ± 34 pA (mean ± S.E.M., n = 60), a capacitance jump (∆Cm) of 365 ± 33 fF (n = 60) and 10.6 ± 1.2 fluorescent A647 spots in ~70–160 μm² of the foot area of the cell (n = 60 cells, Fig. 1b). This stimulation protocol was used because it consistently induces endocytosis (Fig. 1b)15,26.

Three sets of evidence suggest that A647 spots reflect vesicle fusion, owing to A647 diffusion from the bath to open vesicles. First, most spots occurred during and within 1 s after depolarization (Fig. 1c, upper, 60 cells, 636 spots in total). This time course (Fig. 1c, upper) was parallel to the immediate capacitive jump induced by depolarization, which reflects exocytosis (Fig. 1c, lower). The spot number per cell was also proportional to the ∆Cm (Fig. 1d). Second, analogous to calcium-triggered exocytosis, removing extracellular calcium abolished both spot occurrence and ∆Cm (n = 10 cells, P < 0.01, Supplementary Fig. 1). Third, in cells expressing neuropeptide Y-EGFP (NPY-EGFP, granule lumen cargo), 97% (= 175/181) of the NPY-EGFP release events were accompanied with A647 spots (Fig. 1e, arrows). The remaining 3% were likely too small or fast to be resolved. 58 ± 5% of A647 spots (n = 350 spots, 23 cells) overlapped with NPY-EGFP-positive granules and their release (Fig. 1e). The percentage of overlap increased linearly towards 100% as the NPY-EGFP-positive granule density increased (Fig. 1f). Thus, A647 spots that did not overlap with NPY-EGFP-positive granules were due to fusion of NPY-EGFP-negative granules.

Four sets of evidence suggest that the A647 spot at the onset reflects an Ω-profile at the fusion instant. First, under the confocal microscope, the full-width-half-maximum (W1/2) of NPY-EGFP-positive granule (475 ± 13 nm, n = 48 granules) was similar to the overlapping A647 spot’s W1/2 at the onset (490 ± 13 nm, n = 48 spots, P = 0.15, t-test; Fig. 2a). STED imaging (~90 nm resolution) confirmed that the W1/2 of NPY-EGFP-positive vesicle (332 ± 5 nm, n = 278 granules, 7 cells) was similar to the W1/2 of spots (369 ± 10 nm, n = 101 spots, 31 cells) induced by depol1s in different cells bathed with Alexa Fluor 488 (A488, 30–60 μM, Fig. 2b). Note that A488 or EGFP, but not A647 can be used in our STED microscope. The spot W1/2 measured with STED microscopy was close to electron microscopic measurements (~300 nm)13, supporting the idea that fused vesicles do not collapse.

Second, to visualize these structures, we conducted STED imaging at the cell-centre (>2 μm above bottom), where the plasma membrane was approximately perpendicular to the coverslip (Fig. 1a). Depol1s induced A488 spots only adjacent to the cell membrane (Fig. 2c, n = 45). These ‘side’ images showed Ω-like profiles with a pore beyond our resolution (Fig. 2c).

To quantify the Ω-profile, we simulated an Ω-profile with a diameter of 300 nm and a pore <100 nm (Fig. 2d, see Supplementary Fig. 2 for derivation). With a dye outside the cell membrane, line profiles through the simulated Ω-profile centre showed a dip right before reaching the membrane (Fig. 2d, arrow). As expected for an Ω-profile, the dip for a 45° line was larger and wider than a horizontal line (Fig. 2d). These features were not observed in a simulated ‘FC’ profile with an opening that is the same or larger than the vesicle diameter (Fig. 2e, Supplementary Fig. 3).

The image and line features of the simulated Ω-profile were also observed in STED side images of A488 spots (Fig. 2c, arrow), confirming that A488 spots were Ω-profiles. After depolarization,
we did not observe spots that resembled the simulated collapse-like images and line features (see Methods for criteria of side imaging). However, we did observe some resting membrane curvatures (Fig. 2f) resembling the simulated ‘collapse’ image and line features (Fig. 2e). Thus, the lack of ‘collapse’ profiles after depolarization was not due to limited spatial resolution.

Third, the Ω-profile appeared at the spot onset (within 36 ms, our time resolution, 45 spots, see also Figs 3–5). This rapid appearance, together with the overlap between the spot appearance and NPY-EGFP release (Fig. 1e,f), suggests that the Ω-profile is due to fusion, but not slow, endocytic membrane invagination.

Fourth, some spots may close their pore without detectable structural changes (for example, Ω-close fusion, described later), confirming that the Ω-profile’s pore is too small to be resolved.

**Seven modes of Ω-profile change.** To determine how Ω-shaped membrane profiles change in size and pore opening or closing status, we performed confocal imaging (every 5–15 ms) at the cell-bottom with A647 and A488 in the bath being excited strongly and weakly, respectively (A647/A488 experiments, reasons explained later). We found that the structures may change in seven ways described below (636 spots, 60 cells), which reflect different size changes of the Ω-profile (no change, enlarge, shrink partially or completely) and the pore status (opened or closed). These patterns were confirmed with STED imaging at the cell-bottom (STED/cell-bottom, every 26 ms, strong excitation) and cell-centre (STED/cell-centre, every 36 ms, strong excitation).

In 11% of the spots (73/636 spots) in confocal/A647/A488 experiments, A647 and A488 fluorescence intensity (F_{647}, F_{488}) peaked mostly rapidly (<100 ms) and stayed unchanged (<25%) for 30 s (the end of our image recording), during which A647 or A488 spot’s W was stable (A647: 485 ± 8 nm at onset, 474 ± 7 nm 30 s later, n = 73 spots; P = 0.56, t-test; Fig. 3a, Supplementary Movie 1). Similar results were confirmed with STED/cell-bottom imaging: W was 380 ± 35 nm at the onset, and was 400 ± 40 nm 6–8 s later (n = 5; P = 0.74; Fig. 3b). STED data beyond ~8–10 ms after stimulation were discarded, because the whole-cell configuration was often lost due to strong STED depletion laser. STED/cell-centre imaging showed an Ω-profile with a dip in line profiles, which remained stable for 6–8 s (n = 6, Fig. 3c). This pattern is termed Ω-stay with an open pore. The evidence supporting an open pore is explained below.

In 33% of the spots in A647/A488 experiments, F_{647} and F_{488} peaked simultaneously (<70 ms). Subsequently, F_{647} remained unchanged for 0.3–30 s, then decayed monoexponentially to baseline with a τ of 2.9 ± 0.1 s, whereas F_{488} remained unchanged (n = 210 spots, Fig. 3d,e; Supplementary Movie 2). The A647 or A488 spot’s W was also stable: A647 spot’s W at F_{647} peak was 474 ± 5 nm at the F_{647} peak, and was 462 ± 4 nm when F_{647} decayed to 25% of the peak (n = 210, P = 0.18, t-test; Fig. 3d,e). STED/cell-bottom imaging confirmed this result: W was 371 ± 17 nm at the fluorescence (F_{STED}) peak, and was 351 ± 16 nm at 20–30% of F_{STED} peak (n = 27, P = 0.43, Fig. 3f). This pattern, termed here as Ω-stay, reflects Ω-profile pore closure resembling KR, except that closing time can be long. The following two sets of evidence support this conclusion.

First, when F_{647} (strong excitation) but not F_{488} decayed, removing A647 excitation halted F_{647} decay, and resuming excitation recommenced F_{647} decay (n = 11 spots, Supplementary Fig. 4a). After F_{647} decay, increasing A488 excitation increased...
Figure 2 | Resolving the Ω-profile at the fusion instant. (a) Confocal images of a NPY-EGFP-positive granule before release and the A647 spot at the spot onset at the same location (upper). Normalized fluorescence intensity profiles ($F_{\text{norm}}$) from dotted lines are also shown (lower, applies to b). (b) STED images (upper) of a NPY-EGFP granule and an A488 spot at the spot onset (from different cells). Line profiles are also plotted (lower). (c) STED/cell centre images at 0.2 s before (left) and 0.3 s (right) during depo$_{\text{H}}$ (upper). $F_{\text{norm}}$ are also shown for two lines across the spot centre, one perpendicular to the plasma membrane, the other 45° apart (applies to d-f). The arrow indicates the typical feature of the Ω-profile: a dip in the line profile that is wider and larger for the 45° line. Bright fluorescence in the right side of each image represents extracellular A488, whereas dim fluorescence in the left side of each image means the intracellular compartment with no A488 (applies to all plots at the STED per cell-centre setting). (d, e) Simulation showing side images and line profiles (solid and dotted) before (left) and after (right) the appearance of an Ω-profile (d, pore size: 50 nm, vesicle size: 300 nm) or a collapsed profile (e). The arrow in d indicates the typical feature of the Ω-profile: a dip in the line profile which is wider and larger for the 45° line. Images are taken from Supplementary Figs 2f,g and 3c. Simulation methods are described in Supplementary Figs 2,3. (f) Two STED/cell-centre images and line profiles (left, right) that resemble the presumed collapsed profile. Images were obtained in resting conditions.

$F_{\text{488}}$, but followed by a monoexponential decay ($n = 10$ spots, Supplementary Fig. 4b). Thus, strong excitation decreased $F_{\text{467}}$ (or $F_{\text{488}}$). However, strong excitation alone was insufficient to cause $F_{\text{467}}$ decay, because $F_{\text{467}}$ may remain stable (for example, Fig. 3a) or decay at different onsets (for example, Fig. 3d,e). Thus, $F_{\text{467}}$ decay must reflect pore closure, which prevents exchange of bleached dye (caused by strong excitation) with fluorescent dye in the bath. In contrast, a stable $F_{\text{467}}$ (Ω-stay) reflects an open pore (Fig. 3a–c).

Second, as described later, endocytosis reconstructed from Ω-close and other ‘close’ modes matched whole-cell endocytosis. Moreover, block of whole-cell endocytosis by prolonging whole-cell dialysis or inhibition of dynamin abolished ‘close’ fusion. Thus, ‘close’ modes reflect endocytosis.

In 8% of the spots in A647/A488 experiments, $F_{\text{467}}$ and $F_{\text{488}}$ increased in parallel in two phases, initially within ~300 ms and subsequently in ~1–20 s (Fig. 4a). $W_{\text{H}}$ at the onset was 486 ± 8 nm ($n = 52$), similar to Ω-stay (485 ± 8 nm, $n = 73$, $P = 0.78$, t-test) or Ω-close (474 ± 5 nm, $n = 210$, $P = 0.14$). It increased to 600 ± 12 nm at the fluorescence peak ($n = 52$, $P < 0.001$, Fig. 4a). STED/cell-bottom imaging showed similar patterns: $W_{\text{H}}$ at the onset was 399 ± 13 nm ($n = 8$), similar to that of Ω-stay ($380 ± 435$ nm, $n = 5$), $P = 0.60$ or Ω-close ($371 ± 17$ nm, $n = 27$, $P = 0.42$), but increased to 508 ± 26 nm ($n = 8$) at the peak ($P < 0.01$). STED/cell-centre imaging revealed an Ω-profile (with a dip in line profiles) and its enlargement towards the cytosol ($n = 6$ spots, Fig. 4b, Supplementary Movie 3). This mode is termed Ω-enlarge-stay. The slow time course is not due to slow diffusion of the dye into the vesicle, but to the slow size increase, because the initial spot $W_{\text{H}}$ was similar to Ω-stay or Ω-close, and the dye diffuses to a granule in milliseconds$^{27}$.

About 2% of the spots in A647/A488 experiments showed initial changes similar to Ω-enlarge-stay, but followed by $F_{\text{467}}$ decay to baseline with a $\tau$ (2.8 ± 0.2 s, $n = 15$) similar to that of Ω-close while $F_{\text{488}}$ remained unchanged (Fig. 4c). This pattern reflected Ω-profile enlargement and closure, termed Ω-enlarge-close.

In 13% of the spots in A647/A488 experiments, $F_{\text{467}}$ and $F_{\text{488}}$ peaked rapidly (<50 ms) and subsequently decreased in parallel by 50–89% with a $\tau$ of 273 ± 41 ms ($n = 84$), then stayed unchanged for 30 s (Fig. 5a). $W_{\text{H}}$ at the onset was 505 ± 8 nm, similar to that of Ω-stay, but decreased to 359 ± 6 nm at the steady-state ($n = 84$, $P < 0.001$, t-test, Fig. 5a). STED/cell-bottom imaging confirmed the spot shrinkage: $W_{\text{H}}$ decreased from 379 ± 26 to 237 ± 18 nm ($n = 22$, $P < 0.01$, t-test, Fig. 5b, Supplementary Movie 4), and the STED fluorescence intensity ($F_{\text{STED}}$) in the spot’s outer ring decayed faster to a lower value near baseline than the spot’s centre ($n = 22$, for example, Fig. 5b). Consistently, STED/cell-centre imaging showed that the Ω-profile (with a dip in line profiles) shrank towards the plasma membrane without vesicle budding off, then maintained the Ω-profile ($n = 12$ spots, Fig. 5c), termed Ω-shrink-stay.

In 14% of the spots in A647/A488 experiments, $F_{\text{467}}$ and $F_{\text{488}}$ showed initial patterns similar to Ω-shrink-stay, except that after a variable delay (0.3–30 s) in the ‘stay’ phase, $F_{\text{467}}$ decayed to
Figure 3 | Ω-stay and Ω-close fusion. (a-c). Ω-stay. (a) $F_{647}$ (red), $F_{488}$ (green). $W_h$ and sampled images (average of 4) at times indicated (lines) are plotted versus time for a spot at the confocal/A647/A488 setting (cell-bottom). $F_{647}$ and $F_{488}$ were normalized to the mean value before spot appeared (applies to all plots of $F_{647}$, $F_{488}$, and $F_{STED}$). Images were collected every 15 ms. (b) $F_{STED}$ (STED fluorescence intensity). $W_h$ and sampled images (average of 2) at times indicated are plotted versus time for a spot at the STED/cell-bottom setting (60 μM A488 in bath). Images were collected every 26 ms. (c) $F_{STED}$, $W_h$, sampled images (average of 8, side images of the Ω-profile) and their line profiles (normalized to peak, $F_{norm}$) are plotted versus time for a spot at the STED/cell-centre setting. Images were collected every 36 ms. $W_h$ was measured from the profile of a vertical line (not shown, parallel to cell membrane) across the spot centre. Solid and dotted line profiles correspond to solid and dotted lines, respectively. The arrangements in a, b and c apply to all plots in Figs 3-6 at confocal/A647/A488, STED/cell-bottom, and STED/cell-centre setting, respectively. (d-f) Ω-close at confocal/A647/A488 (d, e) and STED/cell-bottom setting (f). Arrows indicate pore closure (apply to ‘close’ fusion in Figs 3–6). E shows two spots (upper, lower) with different pore closing time ($W_h$ and sampled images not shown).

Figure 4 | Ω-enlarge-stay and Ω-enlarge-close. (a,b) Ω-enlarge-stay at confocal/A647/A488 (a) and STED/cell-centre setting (b). (c) Ω-enlarge-close at confocal/A647/A488 setting.
baseline with a τ (2.8 ± 0.1 s, n = 87) similar to that of Ω-close while F_{A88} remained unchanged (Fig. 5d). The spot W_{H} was 509 ± 7 nm at the onset, decreased to 418 ± 5 nm (n = 87, P < 0.001, t-test) at the temporary 'stay' phase, then remained uncharged as F_{A488} decayed (409 ± 6 nm at ~30% of the 'stay' amplitude, n = 87, P = 0.51, t-test, Fig. 5d). STED/cell-bottom imaging showed similar pattern: W_{H} decreased from 356 ± 18 nm at the onset to 244 ± 12 nm (n = 20, P < 0.001, t-test) at the temporary 'stay' phase, then remained stable as F_{STED} decayed to baseline (254 ± 13 nm at ~30% of amplitude at the transition phase, n = 20, P = 0.47, t-test; Fig. 5e). F_{STED} in the spot’s outer ring decayed faster to a lower value than the spot’s centre in the initial shrinking phase, indicating Ω-profile shrinkage (n = 20, for example, Fig. 5e). Consistently, STED/cell-centre imaging showed that the Ω-profile (and the dip in line profiles) initially shrunk towards the plasma membrane, then its size stayed unchanged while F_{STED} decayed to baseline (n = 13 spots, Fig. 5f). These results reflected the Ω-profile shrinkage and closure, termed Ω-shrink-close.

In 18% of the spots in A647/A488 experiments, F_{A488} peaked rapidly (<50 ms), then decreased in parallel to baseline with a τ (1.09 ± 0.11 s, n = 115, Fig. 6a,b) faster than the dye bleaching τ during Ω-close (2.9 ± 0.1 s, n = 210, P < 0.001, t-test). The decay τ was mostly less than 1.5 s, and sometimes only 15–50 ms (Fig. 6b). The W_{H} decreased from 504 ± 12 nm at the onset to 365 ± 10 nm (n = 115) at 23 ± 1% of the peak F_{A488} (for example, Fig. 6a), beyond which W_{H} was too dim to measure. STED/cell-bottom imaging confirmed this pattern: W_{H} decreased from 362 ± 33 nm at the onset to 225 ± 27 nm at 16 ± 2% of the F_{STED} peak (n = 19, P < 0.01, t-test; Fig. 6c, Supplementary Movie 5). F_{STED} in the spot’s outer ring decayed faster than the centre (n = 19, Fig. 6c), confirming Ω-profile shrinkage. STED/cell-centre imaging showed shrinkage of the Ω-profile (and the dip in line profile) without vesicle budding off (n = 8, Fig. 6d, Supplementary Movie 6), termed Ω-shrink.

Figure 5 | Ω-shrink-stay and Ω-shrink-close. (a-c) Ω-shrink-stay at confocal/A647/A488 (a), STED/cell-bottom (b) and STED/cell-centre setting (c). In a, the scale was set to see dim red images, but partly saturate the brightest red image. In b, F_{STED} (peak normalized, F_{STED,n}) in the inner circle (red) and the outer ring (between red and blue circles, blue) are also plotted, showing faster decay of blue trace and thus the spot shrinkage. (c) Left two images, average of two single images; right two images, average of eight single images. (d-f) Ω-shrink-close at confocal/A647/A488 (d), STED/cell-bottom (e) and STED/cell-centre setting (f). In e, F_{STED,n} in the inner circle (red) and the outer ring (between red and blue circles, blue) are also plotted to show spot shrinkage. (f) Left two images, average of two single images; right three images, average of eight single images.

In summary, we described seven ways in which an Ω-shaped membrane profile can change, including three close modes (Ω-close, Ω-enlarge-close, Ω-shrink-close), three stay modes (Ω-stay, Ω-enlarge-stay, Ω-shrink-stay) and Ω-shrink (Fig. 7a). Since we limited imaging to 30 s, pore closure for stay modes beyond 30 s is possible. In addition, we observed occasional events not following these typical patterns. Atypical changes included pore closure and reopening, reflected as F_{A488} bleaching (pore closure) and sudden increase to the original level (pore reopening) while F_{A88} remained unchanged; Ω-stay followed by Ω-shrink or Ω-shrink-stay, reflected as stable F_{A488} value followed by parallel decrease of F_{A487} and F_{A488}; and Ω-enlarge-stay followed by Ω-shrink-stay, reflected as parallel increase and then decrease of both F_{A487} and F_{A488}. These events reflect the continuous nature and flexibility of post-fusion structural changes.

No FC fusion. We did not observe FC fusion (Fig. 7b). The predicted structural change of FC would be spot enlargement while dimming at the cell-bottom, and the collapse of the Ω-profile (Fig. 2e,f) at side images. Neither of these structural
Spot size changes are not caused by movement. The spot enlargement or shrinking we observed was not due to focal plane changes, because while some spots enlarged or shrunk, pre-existing fluorescent structures 1–2 μm away did not change (Supplementary Fig. 5a–c). Could it be due to localized (<1–2 μm) membrane movement that pushes the Ω-shaped membrane profile into the cytosol in the z axis? Four sets of evidence exclude this possibility.

First, the fluorescence within ~0.5–1 μm surrounding the spot remained unchanged at the STED cell-bottom setting (n = 101 spots, for example, Supplementary Fig. 6), indicating no movement surrounding the spot. Similar results were observed using FM4–64 to label membrane (Supplementary Fig. 7a) and Atto 488 to identify fusion modes (n = 75 spots, 6 cells, Supplementary Fig. 7b–d). Second, at the cell-centre setting where the movement to the cytosol could be resolved at the microscopic x/y plane (illustrated in Supplementary Fig. 8c–e), we did not observe any such movements (n = 45 spots; for example, Supplementary Fig. 8f–j). Third, at a z resolution of ~100–150 nm total internal reflection fluorescence microscopy (TIRF, cell-bottom setting, 30 μM Alexa 555 in bath) showed that after depol1s, the spot size may shrunk completely (Ω-shrink, 33 out of 178 spots, Supplementary Fig. 9a), shrink partially (including Ω-shrink-stay and Ω-shrink-close; 33/178 spots, Supplementary Fig. 9b), enlarge (including Ω-enlarge-stay and Ω-enlarge-close; 17/178 spots, Supplementary Fig. 9c), or remain unchanged (including Ω-stay and Ω-close; 95/178 spots, Supplementary Fig. 9d). This result excludes movements to the cytosol of ~350 nm or larger (the confocal z resolution is ± ~350 nm) as the cause for spot size changes. Fourth, to monitor such movements at STED cell-bottom setting (A488 in bath), we switched the focal plane every 70 ms between the cell-bottom (control) and a focal plane that was 300 nm above (upper). Spots induced by depol1s were brighter and focused at the control focal plane, but dimmer and out of focus at the upper focal plane (131 spots). When spots dimmed and shrank completely (28 spots, Supplementary Fig. 10a) or partially (40 spots, Supplementary Fig. 10b) at the control plane, FSTED at the upper plane also decreased, but slightly faster (Supplementary Fig. 10b). Thus, spot shrinking at the control plane is not due to an upward movement towards the cytosol. Similarly, all spots (21 spots) that became brighter and larger at the control plane were also brighter at the upper plane (Supplementary Fig. 10c). In conclusion, the spot size change we observed is not due to movement to the cytosol, but to actual structural changes.

Seven modes observed with other image settings. The seven modes were not only observed with the A647/A488 setting, but also with other settings, which validates our observations. When we used only one dye (A647) and excited it weakly, the spot size could remain the same (n = 42 spots), increase (n = 7 spots), or decrease (to some extent or till undetectable, n = 23 spots; Supplementary Fig. 11; 7 cells), indicating that the spot size changes were not due to photo-toxicity caused by strong excitation. When we excited A488 strongly, but A647 weakly, depol1s induced 62 spots (9 cells) showing six modes (Ω-stay, 9 spots; Ω-close, 12 spots; Ω-enlarge-stay, 6 spots; Ω-enlarge-close, 0 spots; Ω-shrink-stay, 9 spots; Ω-shrink-close, 15 spots; Ω-shrink, 11 spots; Supplementary Fig. 12). When we replaced A647 and A488 with Atto 655 (strong excitation/confocal) and Atto 488 (weak excitation/confocal), depol1s induced 143 spots (7 cells) showing seven modes (Ω-stay, 8 spots; Ω-close, 45 spots; Ω-enlarge-stay, 7 spots; Ω-enlarge-close, 3 spots; Ω-shrink-stay, 19 spots; Ω-shrink-close, 31 spots; Ω-shrink, 30 spots; Fig. 7c). With these settings, the percentage of each mode (obtained from smaller data sets) was roughly similar to those obtained with A647/A488 setting.

Three close modes mediate rapid and slow endocytosis. Three sets of evidence in A647/A488 experiments suggest that close fusion (Ω-close, Ω-shrink-close, Ω-enlarge-close) mediates whole-cell rapid (a few seconds) and slow (tens of seconds) endocytosis. First, if we assigned an upstep at every spot’s onset and a downstep at pore closing time (FSTED decay onset) with an amplitude correction for Ω-shrink-close and Ω-enlarge-close (Fig. 8a, see Methods), the up and downstep interval in close events ranged from 0.3–30 s (n = 312; Fig. 8b), covering both rapid and slow endocytotic time frame.

Second, summation of the up and downsteps from all spots (636 spots) yielded reconstructed net exo- and endocytosis...
(N_{exo–endo}, Fig. 8c, red), which matched approximately the corresponding whole-cell endocytosis (Fig. 8c, black, 60 cells) in both time course and amplitude. The match was also observed when we divided cells into four groups based on capacitance decay: decay to baseline within 15 s (group 1), decay by \( > 80\% \) in 30 s (group 2, not including group 1), decay by \( 30–80\% \) in 30 s (group 3) and decay by \( < 30\% \) in 30 s (group 4, Fig. 8d). \( N_{exo–endo} \) and capacitance decayed rapidly with a \( \tau \) of 2.8 s and 3.9 s, respectively, in group 1; but decayed slowly with a \( \tau \) of 8.1 and 13.8 s, respectively, in group 2. In groups 3–4, \( N_{exo–endo} \) and capacitance changes were similar and did not return to baseline. The fraction that did not decay for \( N_{exo–endo} \) and capacitance was similar in all groups (Fig. 8e). These results suggest that close modes mediate most whole-cell rapid and slow endocytosis.

Third, since prolonged whole-cell dialysis blocks endocytosis\(^{21,29}\), we used this feature to determine whether close modes cause whole-cell endocytosis. In 4 cells, depol induced whole-cell endocytosis and 78\% (53/68 spots) close fusion within 1 min after break-in, but induced no whole-cell endocytosis, and only 3\% close fusion (1/31 spots, Fig. 8f, \( n = 4 \) cells) 6 min later, suggesting that close modes cause whole-cell endocytosis.

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**Figure 7 | Seven fusion modes confirmed by imaging with Atto 655 and Atto 488.** (a, b) Schematic drawings of our model called \( \Omega \)-exo–endocytosis (a, seven modes) and the classical FC and KR model (b). Dotted arrows mean that the transition may or may not take place. (c) Atto 655 fluorescence intensity (\( F_{Atto655} \), red), Atto 488 fluorescence intensity (\( F_{Atto488} \), green) and sampled images (average of 5–10 frames) at times indicated (lines) are plotted versus time for a spot undergoing \( \Omega \)-stay fusion. \( F_{Atto655} \) and \( F_{Atto488} \) were normalized to the mean value before spot appeared. Images were collected every 17–34 ms at the confocal cell-bottom setting with Atto 655 (strong excitation) and Atto 488 (weak excitation) in the bath. (d–i) Similar to (c), but for spots undergoing \( \Omega \)-close (d), \( \Omega \)-enlarge-stay (e), \( \Omega \)-enlarge-close (f), \( \Omega \)-shrink-stay (g), \( \Omega \)-shrink-close (h) and \( \Omega \)-shrink (i).
Calcium influx determines fusion modes. From group 4 to 1, ICa increased from 175 ± 25 pA (n = 21 cells) to 576 ± 61 pA (n = 11 cells, Fig. 8d), close events (three close modes) increased and became dominant, whereas the stay events (three stay modes) and Ω-shrink decreased (Fig. 9a). The correlation between ICa and close fusion (Fig. 9a, red), between ICa and stay fusion (Fig. 9a blue), and between ICa and Ω-shrink (Fig. 9a, black) are causal, because in cells showing large ICa (>450 pA during <10 ms depolarization to +10 mV), when we reduced ICa using a 1-s depolarization to +50 mV (102 ± 8 pA, n = 12 cells, Fig. 9b), close events were nearly fully blocked (3.1%), whereas stay events and Ω-shrink increased (Fig. 9c, open symbols, 12 cells, 64 spots). Thus, large ICa triggered close modes, whereas low ICa promoted stay modes and Ω-shrink.

Dynamin mediates fusion pore closure. In control cells with an ICa >350 pA (mean: 499 ± 54 pA, n = 10 cells), depol1s induced...
whole-cell endocytosis and 73% (48/66 spots) of close fusion (Fig. 9d). With the dynamin inhibitor dynasore (80 μM, 20–30 min) in the bath in cells with an ICa > 350 pA (mean: 515 ± 41 pA, n = 14 cells), depol1s induced nearly no whole-cell endocytosis ( ~10–30% at 30 s after depol1s, Fig. 9d) and only 8% (5/63 spots) close fusion (Fig. 9d). Similarly, including in the pipette solution the dynamin inhibitory peptide (QVPSRPNRAP, 20 μM; Tocris)30, which inhibits dynamin interaction with amphiphysin, largely blocked whole-cell endocytosis and reduced close fusion to 9% (4/45 spots) in cells with an ICa > 350 pA (585 ± 73 pA, n = 6). These results suggest that dynamin mediates fusion pore closure, which in turn mediates whole-cell rapid and slow endocytosis.

Ω-profile retains vesicular membrane protein. To determine whether the Ω-shaped membrane profile, which was observed in all seven modes, holds vesicular membrane proteins from diffusion, we imaged cells expressing a vesicular membrane protein VAMP2 tagged with EGFP (VAMP2-EGFP, weak excitation). A647 (bath, strong excitation) was included for identifying fusion modes (see Methods and Supplementary Fig. 13). Depol1s induced 66 A647 spots (14 cells) that coincided with VAMP2-EGFP spots. VAMP2-EGFP fluorescence (FVAMP2) increased at A647 spot onset owing to the pH increase upon fusion (Fig. 10)31. During Ω-stay, FVAMP2 and spot size remained stable (n = 12 spots, Fig. 10a). During Ω-close, spot size remained stable (n = 13 spots, Fig. 10b). Pore closure lead to FVAMP2 decay, whereas FVAMP2 decreased partially in 30 s (n = 13 spots, Fig. 10b), consistent with cavicapture that takes 100 s or longer for re-acidification15,31. During Ω-enlarge-stay, FVAMP2 remained stable, whereas VAMP2-EGFP spot size increased (n = 5, Fig. 10c). During Ω-shrink-stay, FVAMP2 decrease (to 20 ± 2%, n = 9) in the shrink phase was not accompanied by parallel FVAMP2 decrease, but a delayed and smaller FVAMP2 decrease (to 67 ± 7%, n = 9, for example, Fig. 10d). However, VAMP2-EGFP spot Wt decreased in parallel with A647 spot Wt (for example, Fig. 10d, n = 9), suggesting that the Ω-shaped membrane prevents or slows down diffusion of vesicular membrane proteins.

For Ω-shrink-close, A647 and VAMP2-EGFP spot size reduced in parallel (for example, Fig. 10e, n = 5). FVAMP2 did not decay during the shrink phase just like Ω-shrink-stay, but decayed with a delay slowly (n = 5, Fig. 10e), likely due to slow re-acidification15,31. During Ω-shrink, FVAMP2 decreased to baseline (n = 22). If shrinking was rapid, a VAMP2-EGFP diffusion cloud was observed (Fig. 10f). If shrinking was slow, VAMP2-EGFP spot size reduced in parallel with the A647 spot size (Fig. 10g). FVAMP2 reduction significantly lagged behind FVAMP2 reduction, reminiscent of the delay observed in Ω-shrink-stay and Ω-shrink-close (Fig. 10d,e). Thus, in all seven modes, Ω-shaped membrane profiles may prevent or slow down diffusion of vesicular membrane proteins.

At ~20 s after the spot appeared, reducing the bath pH to 5.5 by applying MES solution decreased the pH-sensitive FVAMP2 (ref. 31) to baseline for stay events (including Ω-stay, Ω-enlarge-stay and Ω-shrink-stay, n = 9 spots,Fig. 10h, left), but not for close events (including Ω-close and Ω-shrink-close, n = 8 spots, Fig. 10, right), confirming the pore open or close status, as determined by A647 imaging.

Discussion

We establish a new exo–endocytosis model, termed Ω-exo–endocytosis (Fig. 7a), where the Ω-profile fusion does not dilate, but changes in seven patterns through size transformation and fusion pore closure. This model is fundamentally different from the classical FC/KR model. FC is redefined as Ω-shrink, which merges fused vesicles with the plasma membrane by shrinking.

Figure 9 | Strong calcium influx triggers dynamin-dependent close fusion modes and low calcium promotes stay modes and Ω-shrink. (a) The percentage of CloseSum (including all three close modes), StaySum (including three stay modes) and Ω-shrink plotted versus the mean ICa in four groups described in Fig. 8d (stimulation: depol1s to +10 mV). The percentage was calculated within each group. (b) Sample ICa and Cm induced by a 1 s depolarization to + 50 mV. This cell showed an ICa of ~ 500 pA during a 10 ms depolarization to + 10 mV (not shown). (c) Re-plotting (a) (solid symbols), but including data similar to those shown in (b) (open symbols), where 1 s depolarization to + 50 mV induced the smallest ICa as compared with the mean ICa induced by depol1s to +10 mV in groups 1–4. (d) The mean Cm change (upper, ΔCm) and the percentage of close fusion (CloseSum, lower, including Ω-close, Ω-shrink-close and Ω-enlarge-close) induced by depol1s, in control (10 cells, 66 spots, left) and in cells bathed with 80 μM dynasore (14 cells, 63 spots, right). In both groups, cells with an ICa > 350 pA were selected for analysis.

Figure 10 | Ω-profile retains vesicle membrane protein VAMP2. (a–g) FVAMP2 (red), FVAMP2 (green), Wt of A647 (red) and VAMP2-EGFP (green) spot, and sampled A647 (red) and VAMP2-EGFP (green) images (at times indicated with lines) for spots undergoing Ω-stay (a), Ω-close (b), Ω-enlarge-stay (c), Ω-shrink-stay (d), Ω-shrink-close (e) and Ω-shrink (f): rapid shrinking, diffusion cloud; g: slow shrinking, size reduction observed. Cells were expressed with VAMP2-EGFP and stimulated by depol1s with A647 in the bath. Wt is not measured in f, because VAMP2-EGFP rapidly diffused into a cloud, which did not reflect the Ω-shaped membrane profile size. VAMP2-EGFP spots appeared slightly (~50–100 nm in Wt) larger than corresponding A647 spots (for example, Fig. 10a,b), because VAMP2-EGFP was located at the membrane, whereas A647 was inside the Ω-shaped structure. (h) The FVAMP2 changes in response to a bath pH change from 7.4 to 5.5 (upper) for spots undergoing Ω-stay (left) and Ω-close (right).
but not dilating the Ω-shaped membrane profile. KR is redefined as close fusion, including Ω-close, Ω-shrink-close and Ω-enlarge-close, which may generate different-sized vesicles. Close fusion is triggered by strong calcium influx and requires dynamin to close the pore. Close fusion is the dominant mechanism mediating whole-cell rapid and slow endocytosis. It may also regulate vesicle size by shrinking or enlarging the Ω-shaped membrane profile before fusion pore closure. Consequently, the enlargement
(Ω-enlarge-close) may mediate bulk endocytosis, a form of endocytosis defined as formation of large vesicles from the plasma membrane\textsuperscript{56}. While strong calcium influx triggers close fusion, weak calcium influx facilitates stay fusion (Ω-stay, Ω-shrink-stay, Ω-enlarge-stay) and Ω-shrink. Unlike the irreversible FC model, transition between the different modes in the Ω-exo–endocytosis model is flexible (Fig. 7a). Finally, most vesicular membrane proteins are maintained in the Ω-shaped membrane during various modes of fusion by a mechanism that needs further investigation.

Our model may account for most observations previously interpreted with FC/KR model. For example, Ω-shrink not only accounts for the merging of fused vesicles with the plasma membrane previously attributed to FC, but also allows for the flexibility to close the fusion pore anytime during shrinking (Fig. 7a). The pore of Ω-shrink fusion is much larger than 4 nm, because Ω-shrink released the ~4 nm NPY-EGFP\textsuperscript{52} rapidly with a τ of 33 ± 4 ms (n = 49 spots, for example, Fig. 1e). Ω-shrink with a large pore may explain all live-cell data previously interpreted as FC, such as rapid content release, fusion pore conductance increase above a detection limit (corresponds to ~3–5 nm pore), and disappearance of fusion-generated vesicular images in endocrine cells and neurons\textsuperscript{13,15,25,27,31,33–37}.

Could Ω-shrink replace FC at synapses? FC was first suggested by Heuser and Reese\textsuperscript{2} based on freeze-fracture electron microscopic observation that there are more large openings (diameter: 60–120 nm) at 50 ms than at 3–5 ms after stimulation at neuromuscular junctions\textsuperscript{38}. It predicts that as pore dilates, large openings become dominant. However, large openings are not dominant at any time measured\textsuperscript{2}. Using similar techniques and preparation, Ceccarelli et al\textsuperscript{17} questioned whether FC exists\textsuperscript{39,40}. A recent study shows a widened neck of an Ω-profile consistent with the FC model\textsuperscript{41}. However, FC has not been observed in live cells. An increased surface area\textsuperscript{42} and release of ~15 nm quantum dots\textsuperscript{43} at live retinal and hippocampal nerve terminals supports FC, but can also be interpreted with Ω-shrink that has a large pore. Further work is needed to confirm Ω-shrink fusion in live synapses.

By comparing endocytosis reconstructed from 636 fusion events with concurrently measured whole-cell endocytosis, we found that fusion pore closure during close fusion mediates most slow endocytosis within 30 s after stimulation (Fig. 8c,d, group 2–4). We cannot exclude classical endocytosis beyond 30 s, although closure of stay fusion may also contribute to whole-cell endocytosis. Our finding challenges the traditional view that slow endocytosis is mediated by classical endocytosis.

Can close fusion contribute to slow endocytosis at synapses? Inhibition of slow endocytosis by blocking clathrin-dependent endocytosis\textsuperscript{5} and the existence of a readily retrievable pool\textsuperscript{44–46} argue against this possibility. However, it remains unclear whether close fusion is clathrin-dependent and whether stay fusion generates the readily retrievable pool. Further study is needed to determine whether close fusion contributes to slow endocytosis at synapses.

Rapid endocytosis in endocrine cells and neurons is hypothesized to be caused by KR\textsuperscript{18,19,47}, rapid classical endocytosis\textsuperscript{16,48} or bulk endocytosis\textsuperscript{24,49}. Which mechanism mediates rapid endocytosis remains unresolved, because each endocytic mode’s contribution had not been reconstructed for comparison with whole-cell endocytosis. Providing such comparison for the first time, we found that close fusion (three close modes), which includes bulk endocytosis (mediated by Ω-enlarge-close), underlies rapid endocytosis (Fig. 8d, group 1).

The three close modes provide a mechanism to regulate vesicle size. Hence, they may contribute to or cause vesicle size variation observed within a cell and among different cells\textsuperscript{50,51}. Since vesicle size is proportional to quantal size\textsuperscript{50,51}, regulation of three close modes may in turn modulate quantal size, which defines exocytosis strength, such as increased vesicle size and synaptic strength observed after animal activity\textsuperscript{52}. Previous studies suggest that low calcium concentration triggers rapid KR\textsuperscript{17,18}, which predicts rapid whole-cell endocytosis when calcium influx is reduced or buffered. In contrast, reducing or buffering calcium influx abolishes endocytosis in chromaffin cells and neurons\textsuperscript{20,22,24,53,54}. The present work may explain this discrepancy, because in studies suggesting that KR is triggered by low calcium, an indirectly detected Ω-profile, but not pore closure is interpreted as KR\textsuperscript{17,18}. Such an interpretation is analogous to low calcium-facilitated stay modes reported here (Fig. 9). We found that strong calcium influx triggers close fusion to mediate whole-cell endocytosis, consistent with the finding that calcium influx triggers whole-cell endocytosis (Figs 8,9)\textsuperscript{20–24,53,54}.

Our finding that strong calcium influx triggers close fusion is apparently consistent with results showing that KR is dominant at 90 mM extracellular calcium\textsuperscript{24}. However, increasing extracellular calcium (50 mM) decreases ICa during depolarization\textsuperscript{13}, implying that prolonged extracellular, but not intracellular calcium increase facilitates KR. Consistent with this implication, prolonged intracellular calcium increase does not promote KR\textsuperscript{33}. Taken together, we conclude that strong calcium influx induced by transient depolarization triggers close fusion.

Rapid KR (within seconds) is proposed as a simple reversal of fusion pore opening without dynamin involvement, whereas cavicapure (slow KR) may require dynamin\textsuperscript{1,56,57}. The present work provides experimental data showing that not only slow, but also rapid fusion pore closure is mediated by dynamin (Fig. 9d). Our finding that close fusion is a dominant endocytic mechanism seems in contrast to the infrequent KR observed in cell-attached recordings. This difference is likely due to different definitions and recording conditions. In cell-attached recordings, KR is detected as equal sized capacitance up- and down-steps that occur within 2 s\textsuperscript{7,35,37,58,59}, which would exclude Ω-shrink-close, Ω-enlarge-close and Ω-close that closes after 2 s. Furthermore, close modes were triggered by strong calcium influx during transient depolarization (Fig. 8), whereas KR in cell-attached recordings is often detected at rest or with high potassium depolarization\textsuperscript{13,14,35,37,59} which does not promote close fusion.

How the granule dense core copes with structural changes during Ω-exo–endocytosis is unknown. It might be squeezed out or dissolved first and then released during Ω-shrink\textsuperscript{60,61}, but might stay as observed in pituitary lactotrophs\textsuperscript{61} during stay or close fusion.

In summary, the Ω-exo–endocytosis model may explain most live-cell data previously interpreted with FC/KR model. It may apply to large vesicles in many other cell types, such as pancreatic cells, adipocytes, blood cells, glial cells and neurons that secret dopamine, peptides and hormones\textsuperscript{62}. Whether it applies to small synaptic vesicles deserves consideration, because neuroendocrine chromaffin cells and nerve terminals are similar in many aspects that were traditionally interpreted with classical exo- and endocytosis models, such as capacitance upstrokes and flickers, rapid and slow content release, calcium-triggered rapid and slow endocytosis, bulk endocytosis and proteins involved in exo- and endocytosis\textsuperscript{1,4,5,10,11,20,23,24,63}.

Vesicle fusion has been imaged by many techniques, including TIRF\textsuperscript{31} or polarized TIRF imaging\textsuperscript{64}, two-photon imaging with extracellular dye that our technique is based on\textsuperscript{25}, and interference reflection microscopy\textsuperscript{42}. Using confocal and STED microscopy, we achieved the highest spatial and temporal resolution currently available, ~90 nm/5–15 ms. We detected pore closure by differential excitation of two dyes at a temporal
resolution (~0.3 s) much faster than other imaging methods. Without relying on protein overexpression, our method is much more efficient in capturing vesicle fusion (Fig. 1e,f). Our imaging method opens the door for studying the mechanisms that generate and regulate the Ω-profile, or more generally, membrane curvature.

**Methods**

**Primary bovine chromaffin cell culture.** We prepared primary chromaffin cell culture as described previously. In brief, fresh adult (21–27 months old) bovine adrenal glands (local abattoir), were immersed in pre-chilled 1×Lock’s buffer on ice containing: NaCl, 145 mM; KCl, 5.4 mM; NaH2PO4, 2.2 mM; Na2HPO4, 2.2 mM; glucose, 5.6 mM; HEPES, 10 mM; pH 7.3 adjusted with NaOH. Glands were perfused with 1×Lock’s buffer, then infused with Lock’s buffer containing collagenase P (1.5 mg ml-1, Roche), trypsin inhibitor (0.325 mg ml-1, Sigma) and bovine serum albumin (5 mg ml-1, Sigma) and incubated at 37 °C for 20 min. The digested medulla was minced in Lock’s buffer and filtered through a nylon mesh. The filtrate was centrifuged (39,000 rpm, 10 min), re-suspended in Lock’s buffer and re-centrifuged until the supernatant was clear. Final cell pellet was re-suspended in pre-warmed DMEM low-glucose medium ( Gibco) supplemented with 10% fetal bovine serum (Gibco) and plated onto polystyrene (0.005% w/v, Sigma) and laminin (4 μg ml-1, Sigma)-coated glass coverslips. The cells were incubated at 37 °C with 8% CO2 and used within 1 week. Before plating, some cells were transfection by electroporation (2 μg plasmid DNA containing NPY-EGFP or VAMP2-EGFP) using Basic Neuron Nucleofector Kit (Lonza, Program O-005).

**Electrophysiology.** At room temperature (22–24 °C), whole-cell voltage clamp and capacitance recordings were performed with an EPC-10 amplifier together with the software pCLAMP (Molecular Devices, CA, USA). The holding potential was −80 mV. The frequency of the sinusoidal stimulus was 1,000–1,500 Hz with a peak-to-peak voltage ≤ 50 mV. The bath solution contained 125 mM NaCl, 10 mM glucose, 10 mM HEPES, 5 mM CaCl2, 1 mM MgCl2, 4.5 mM KCl, 0.081 mM TTX and 20 mM TEA, pH 7.3 adjusted with NaOH. The pipette (3–6 MΩ) solution contained 130 mM Cs-glutamate, 0.5 mM Cs-EGTA, 12 mM CsCl, 30 mM HEPES, 1 mM MgCl2, 2 mM ATP and 0.5 mM GTP, pH 7.2 adjusted with CsOH. These solutions phenologically isolated calcium currents.

**Imaging.** With an inverted confocal microscope (TCS SP5II, Leica, Germany, 100× oil objective, numerical aperture: 1.4), A647 (30 μM in bath, Invitrogen) and A488 (30–60 μM in bath, Invitrogen) were excited by a HeNe laser at 633 nm (maximum power: 20 mW) and an Argon laser at 488 nm (maximum power: 25 mW), respectively. Unless mentioned otherwise, the 633 nm laser was set at 60% of the maximum power, whereas 488 nm laser was set at 1.5–2%. A647 fluorescence was collected with a photomultiplier at 643–700 nm, whereas A488, with a GaAsP hybrid detection system at 498–580 nm. The quantum efficiency of the hybrid detection system is two times higher than the photomultiplier, which improved the signal-to-noise ratio for A488 imaging. Both excitation and fluorescence collection were performed simultaneously. Without relying on protein overexpression, our method is much more efficient in capturing vesicle fusion (Fig. 1e,f). Our imaging method opens the door for studying the mechanisms that generate and regulate the Ω-profile, or more generally, membrane curvature.

**Identifying fusion modes with a single dye.** When A647 (strong excitation) and A488 (weak excitation) were imaged (Fig. 10), most fusion modes observed with the StED (signal, time, and distance) microscope could be readily distinguished from F647 change alone, as shown in Figs 3–6. Although F647 decayed in both Ω-shrink and Ω-close fusion, the decay τ for Ω-shrink (1.09 ± 0.11 s, n = 115 spots, Supplementary Fig. 13a) was much faster than that of Ω-close (2.9 ± 0.1 s, n = 210 spots, Supplementary Fig. 13b). Since the decay, τ distribution for Ω-shrink overlapped little with that of Ω-close (Supplementary Fig. 13c), we classified spots with a decay τ < 1.7 s as Ω-shrink. This criterion excluded Ω-close, because the decay τ for all Ω-close events was > 1.7 s (Supplementary Fig. 13). Spots with a decay τ > 2 s were classified as Ω-close, in which 8% could be due to Ω-shrink owing to the overlap distribution of the decay τ (Supplementary Fig. 13c). Such a small error should not significantly affect our main conclusion.

With a single dye (A488, strong excitation) in STED imaging, we identified the fusion modes based on changes in FSTED and Wst close to confocal imaging. STED imaging was more sensitive in detecting the Wst change, making it easier to distinguish between Ω-shrink and Ω-close.

**Exo- and endocytosis reconstruction.** In confocal/A67/4A88 experiments, we labelled each spot at the spot onset as an upstroke with an amplitude of 1, and a downstroke only for three ‘close’ modes at the pore closure time, that is, the onset of A647 bleaching while A488 remained unchanged (Fig. 8a). The downward amplitude was corrected for Ω-shrink-close and Ω-enlarge-close by raising the relative fluorescence changes during the ‘shrink’ or ‘enlarge’ phase to a power of 0.75, which was approximately proportional to the granule surface area or capacity. This correction was based on our observation that the fluorescence change was approximately proportional to the Wst change raised to a power of 3. Here we did not consider granule size differences for each fusion mode, because the mean spot Wst at the fusion onset was similar for seven fusion modes.

**Measuring the pore closure time.** For close modes, the onset of the F647 decay when F488 remained unchanged was taken as pore closure time. The F647 decay time constant was 2.9 ± 0.1 s (n = 210), meaning that at 0.3 s, F647 decays by 10%, which could be well resolved (for arrows in Figs 3d–f, 4d and 5d–f). Thus, our time resolution for pore closure was 0.3 s. If pore closure was less than our resolution, we assigned an arbitrary value of 0.3 s.

**Data selection for analysis.** The data within the first 2 min after whole-cell break-in were excluded. To avoid whole-cell rundown (see also Fig. 8d), cells showing prominent endocytosis overshoot were discarded, because we focused on determining whether ‘close’ modes are responsible for compensatory endocytosis, and the overshoot often curtailed the capacitance increase to a negative value, making it difficult to compare whole-cell exo–endo with reconstructed exo–endocytosis.

**Statistics.** The statistical test used is t-test or paired t-test. The data were expressed as mean ± s.e.m.

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**Author contributions**
H.-C.C. and W.S. did most of the experiments, designed the experiments and participated in the writing. W.-D.Z., E.H., J.S., M.B. and P.J.W. did some experiments. A.J. and F.M. were involved in setting up optical systems and initial chromaffin cell cultures. L.-G.W. designed the experiments and wrote the manuscript.

**Additional information**
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Erratum: Post-fusion structural changes and their roles in exocytosis and endocytosis of dense-core vesicles

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The right-hand side of Supplementary Fig. 13 was inadvertently deleted in the published Supplementary Information file for this Article. The correct version of the figure appears below.
