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The mystery of the fusion pore

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Release of neurotransmitters occurs by opening of a fusion pore thought to be formed by action of SNARE proteins but if the fusion pore is a lipidic or proteinaceous structure is controversial. A new study employing very small nanodiscs shows that it is both.

Membrane fusion is of central importance in all eukaryotic cells from the biosynthetic pathways to exocytotic secretion of a wide range of compounds. The SNARE (Soluble NSF Attachment REceptor) complex, which in mammalian neurons and neuroendocrine cells is composed of the proteins synaptobrevin-2 (Syb2 also called VAMP2), syntaxin-1 (Stx1), and SNAP-25, plays a central role in this process¹. The vesicular protein (v-SNARE) Syb2 is a 116 amino acid protein anchored in the vesicle membrane by a single transmembrane (TM) domain. Stx1 is correspondingly anchored in the plasma membrane via a single TM helix. The third component, SNAP-25 has lipid anchors in the plasma membrane. SNAP-25 and Stx1 are called t-SNAREs, being in the target membrane for fusion of secretory vesicles. When reconstituted into liposomes, these proteins represent a minimal machinery that promotes fusion²⁻⁴, which has led to the hypothesis that the SNARE proteins open the fusion pore that allows vesicular contents to be released into extracellular space. Electrophysiological measurements of fusion pore conductance revealed that the initial fusion pore in neuronal cell types has molecular dimensions with an estimated typical diameter of 1-2 nm⁵. However, the molecular structure of the fusion pore is still a mystery. It is not known how many SNARE complexes participate in fusion pore formation⁶ and if the fusion pore channel is lipidic⁷, proteinaceous,⁸ or of proteolipid composition⁹.

Bao et al (XXX) address this question using very small nanodiscs. Nanodiscs are self-assembled particles, which contain a single phospholipid bilayer with nanometer dimensions stabilized by an encircling membrane scaffold protein (MSP)¹⁰. Fusion between nanodiscs with ~13 nm diameter incorporating Syb2 and small unilamellar vesicles containing the t-SNAREs Stx1 and SNAP-25 had recently been demonstrated by Shi et al.¹¹. Bao et al now incorporated Syb2 into nanodiscs as small as 6 nm, which appears too small to accommodate a lipidic fusion pore (Fig.1). However, in spite of their small size they do fuse with t-SNARE containing vesicles as inferred from fluorescence dequenching indicating lipid mixing and release of glutamate encapsulated inside the liposomes indicating formation of a pore. In the lipid mixing assay the fluorescence signal is in part protected from dithionite quenching. This indicates that full fusion associated with transfer of fluorescent

lipid from the nanodisc to the intravesicular leaflet, followed by closure of some of the fusion pores that had formed.

If fusion pores cannot be lipidic as concluded from the small nanodisc size, they may be formed by protein transmembrane domains like an ion channel or gap junction pore. The role of transmembrane domains in forming a pore has been investigated in ion channel research for many years using cysteine scanning and labeling using hydrophilic methanethiosulfonate reagents¹². Residue locations that are labeled are accessible from the aqueous phase and line the ion channel pore. Bao et al use this approach to probe the fusion pore. They find that Syb2 TM domain mutants V101C, I105C, and I109C are labeled in the presence of t-SNARE liposomes but not in their absence and conclude that during fusion these residues are accessible and therefore line the fusion pore. Since 6 nm nanodiscs have very few lipids raise the possibility that they may not be able to shield the TM domains from solvent entirely, the Syb2 TM mutants V101W and I105W also show somewhat reduced glutamate release suggesting that these might indeed be facing the fusion pore. Could the pore be formed by rings of SNARE TM domains?

This seems also unlikely because fusion was readily observed in their experiments with nanodiscs containing as few as 2 copies of Syb2. Two v-SNAREs are too few to form a proteinaceous pore lined by Syb2 TM domains (which would require at least 3 TM domains) and the question arises how can a fusion pore be formed that is neither lipidic nor formed by a protein transmembrane channel. The likely answer is that the fusion pore must be of a hybrid composition incorporating protein as well as lipids and that both, SNARE TM domains and lipids line the pore. But if a lipidic fusion pore cannot be accommodated by a 6 nm nanodisc, what would the structure of such a proteolipid fusion pore look like?

Molecular dynamics simulations of SNARE mediated membrane fusion of small vesicles have recently provided interesting insight into structural aspects of fusion pore formation¹³. Fig. 2A shows a possible arrangement of a nanodisc docked to a membrane by 4 SNARE complexes. A coarse grain simulation of this system led to fusion pore formation after $\sim 1 \mu\text{s}$ and a simulation snapshot at $\sim 1.7 \mu\text{s}$ simulation time (Fig. 2B) shows a water filled fusion pore traversing the membrane and the nanodisc. The fusion pore is mostly lined by lipid head groups but also incorporates the C termini of the TM domains of Syb2 and Stx1 consistent with previous simulation results¹³. It seems possible that a similar fusion pore could be formed with the small 6 nm nanodisc. If the fusion pore structures obtained in such simulations are consistent with the cysteine and tryptophan scanning data will be interesting to investigate

Given that the experiments by Bao et al were performed in a reconstituted system, the question arises how these results and conclusions relate exocytotic fusion in cells and synapses. The question how many copies of Syb2 are needed for fusion has previously been addressed in cultured hippocampal neurons from Syb2/cellubrevin double knock-out mice. In these neurons, expression of Syb2 fused to pHluorin, a pH-sensitive variant of GFP, at its luminal C terminus (spH) rescued fusion if 2 copies were present on a vesicle¹⁴. It thus appears that the requirement of just two copies of Syb2 for fusion pore formation applies to the in vitro nanodisc-SUV system as well as synaptic vesicles.

Does this mean that fusion is achieved by the action of two SNARE complex bridging the vesicle and plasma membranes? It is well known that syntaxin forms clusters in the plasma membrane of endocrine cells¹⁵. These clusters appear to assemble during docking and are required for docking and disassemble after fusion^{16,17}. In PC12 cells the t-SNARE clusters consist of 50-70 molecules¹⁸ and in INS1 cells of ~30; in neurons they consist of at least 10 but possibly many more¹⁹. Experiments in chromaffin cells performed with a SNAP-25 mutant affecting fusion kinetics indicated that for fast fusion at least 3 copies of SNAP-25 are needed, possibly more²⁰. This points to the possibility that the requirements for v-SNAREs and t-SNAREs might be different such that only 2 copies of Syb2 are needed but tens of t-SNARE copies.

If this were the case, why then is it that synaptic vesicles contain tens of Syb2 molecules, a number close to that in t-SNARE clusters? Also it appears that in reconstituted systems the lipid mixing efficiency was maximal with small (40 nm) liposomes with only one synaptobrevin, but 23-30 copies were required for efficient lipid mixing in large (100 nm) liposomes²¹, suggesting that cooperativity between SNARE complexes is variable and depends on vesicle size or membrane curvature that could also be influenced *in vivo* by proteins such as synaptotagmin. Bao et al used nanodiscs where the effective membrane curvature is hard to estimate. In the previous study using larger nanodiscs it was reported that 1 copy of Syb2 was sufficient to open a fusion pore but 3 copies were required for efficient fusion pore expansion. In reconstituted systems SNARE requirements clearly depend on lipid composition and cholesterol⁴ and the likely influence of leaflet asymmetry could not yet be addressed in reconstitution experiments.

In summary, the model where SNARE/lipid complexes form proteolipid fusion pores⁹ is receiving strong support from the accumulating evidence that as few as two Syb2 copies are sufficient for fusion pore formation. But the functional properties of such fusion pores are still to be determined. The large numbers of Syb2 molecules on the vesicle and of t-SNAREs in the clusters on the plasma membrane is likely of functional significance. Recent evidence suggests that t-SNARE clusters may be needed for rapid fusion pore expansion²². More detailed future investigations of fusion pore properties formed by action of variable numbers of SNAREs might further illuminate the mystery of the fusion pore.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A lipidic fusion pore (center) might fit a 12 nm nanodiscs (top) but not a 6 nm nanodisc (bottom). Nanodiscs were simulated using GROMACS 4.6²³ using Martini force field²⁴. The structure of ~12nm MSP1E2 was modeled based on crystal structure 1AV1 of the lipid binding domain of ApoA-I. To generate the smaller 6 nm disc helix 4 to helix 6 were deleted using Modeler. The lipids were chosen based on the synaptic vesicle lipid composition (12 nm disc: 154 CHOL, 13 PPCS, 69 POPC, 89 POPE and 25 POPS; 6 nm disc: 50 CHOL, 5 DPSM (sphingomyelin), 23 POPC, 30 POPE, 9 POPS). Lipid numbers were chosen based on simulation results showing that MSP1E2 nanodisc contains ~125 DMPC lipids/leaflet²⁵. DMPC has an area per lipid (APL) of 0.61 nm². Considering the average APL of 0.44 nm² for the multicomponent planar bilayer, total number is ~173 lipids/leaflet. The appropriate lipids from a pre-equilibrated asymmetric bilayer were placed in the empty nanodisc. A short equilibration (50 ns) was carried out with the head groups restrained along Z-direction (normal to bilayer) followed by an unrestrained 500 ns simulation.

Fig. 2.
(A) Possible arrangement of a 12 nm nanodisc docked to a membrane via 4 SNARE complexes after 20 ns simulation time. (B) Fusion pore snapshot after 1.665 μ s simulation time with the isodensity surface of waters in the pore in side view. Colors: Syb2 – blue, Stx1 – red, SNAP-25 – green, MSP – yellow.