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Research Highlight

Counting the SNAREs needed for membrane fusion

Geert van den Bogaart and Reinhard Jahn*

Department of Neurobiology, Max-Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany
* Correspondence to: Reinhard Jahn, E-mail: rjahn@gwdg.de

SNARE proteins mediate most membrane fusion reactions in eukaryotic cells, and are thereby vital for many functions, including synaptic transmission, cell growth, and cytokinesis. However, until recently it was still unclear how many of these proteins are required to catalyze membrane fusion *in vivo*. In a recent *Science* paper, Mohrmann et al. (2010) elegantly show that in chromaffin cells, three SNARE complexes are required for the synchronous release of granules, whereas only a single SNARE complex is sufficient for overall release.

In neuronal exocytosis, small membrane vesicles fuse with the cell membrane, thereby releasing their neurotransmitters in the synaptic cleft. This fusion reaction is mediated by a small well-conserved group of membrane-anchored proteins that possess a domain of 60–70 amino acids, called SNARE proteins. Complementary sets of those SNAREs are present in both the fusing membranes. They assemble *in trans*, forming a very stable four-helix coiled-coil bundle (so-called 'zippering'), which brings the membranes together and initializes membrane fusion (Jahn and Scheller, 2006). However, it was still unknown how many of these SNARE complexes are required to catalyze membrane fusion. Several studies addressed this question using a wide range of both *in vitro* and *in vivo* approaches.

Membrane fusion can be mimicked *in vitro* by mixing two populations of artificial membranes containing complementary SNAREs. Such systems allow for precise control of the reaction conditions and are therefore ideal to determine the minimal number of SNAREs required for membrane fusion. It was recently inferred that 3–9 SNARE complexes are needed for membrane fusion, based on a multi-step kinetic model and assumed that these steps corresponded to the number of SNARE complexes (Domanska et al., 2009). In a more direct approach, the fusion efficiency was measured as a function of the SNARE density and here membrane fusion required 5–11 SNARE complexes (Karatekin et al., 2010). In contrast, using artificial membranes with very low SNARE densities, we recently showed that only a single SNARE complex is sufficient for membrane fusion of small artificial vesicles (van den Bogaart et al., 2010). These different numbers reported in these studies might represent differences in the precise reaction conditions, such as membrane curvature or the lipid composition. Overall, this reflects the single big disadvantage of *in vitro* approaches: it is impossible to completely mimic the reaction conditions of the cell. Thus, although *in vitro* work allows us to determine the number of SNARE complexes required for membrane fusion under specific conditions, it is always unclear whether this number will actually reflect the requirements in a cell because the reaction conditions will be inherently different.

Several studies in various cellular systems were performed to overcome the limitations of *in vitro* approaches (Montecucco et al., 2005). Based on a competitive inhibition experiment in cracked PC12 cells (a neuroendocrine tumor cell line) with soluble SNARE domains, it was concluded that three SNARE complexes were required for vesicle release (Hua and Scheller, 2001). More recently, in a study employing mutant SNAREs in intact PC12 cells, it was inferred based on steric hindrance that 5–8 SNAREs were required for membrane fusion (Han et al., 2004). Lastly, an even higher estimate of 10–15 SNARE complexes was reported in spinal cord neurons using botulinum neurotoxins that cleave SNAREs through specific proteolysis (Keller et al., 2004). Thus, the number of SNARE complexes catalyzing membrane fusion was estimated to range from 3 to 15 and was not known with a very high certainty. Although this wide range might reflect variation among cellular systems and fusion reactions, it can perhaps more likely be explained by the difficulty to accurately determine the number of SNAREs capable of fusion after inhibition. This problem is now tackled by Mohrmann et al. (2010) in a very cunning way.

Mohrmann et al. (2010) expressed a mutant of one of the SNAREs, SNAP-25, that was unable to catalyze membrane fusion, but was still able to form the tight coiled-coil complex. Thereby, this mutant effectively formed a dead-end complex, which resulted in a reduction of the number of active SNAREs actually available for membrane fusion. This mutant was then co-expressed together with wild-type SNAP-25 in knock-out chromaffin cells. They employed an expression system that resulted in different levels of active versus inactive SNAP-25 per cell. To determine these ratios, they fused both proteins to spectrally separate fluorescent proteins and employed fluorescent microscopy. Subsequently, the granule release of each cell was characterized with conventional electrophysiology. Hence, they were able to accurately determine the effect on exocytosis while reducing the available active SNAREs. For fast

synchronous release, they obtained a third power dependency on the number of SNAREs, whereas slower overall release showed a linear dependency. This indicates that three SNARE complexes are required for fast evoked release, whereas a single is sufficient for the slower phase of exocytotic release. Although these data agree well with the study of Hua and Scheller (2001) in cracked PC12 cells and our work with small artificial membranes (van den Bogaart et al., 2010), it is surprisingly low when considering other previous reports. In this respect, a very recent single molecule pH-fluorin study by Sinha and Klingauf (personal communication) in cultured neurons can be proved of high importance. Here, a system was developed to directly count the number of SNAREs participating in vesicle release by means of fluorescence. They found that only two SNARE complexes can completely rescue evoked release of synaptic vesicles in neurons derived from knockout mice, i.e. thus being very similar to that determined by Mohrmann et al. (2010). These low numbers are incompatible with current popular models, where the fusion pore is surrounded by multiple SNARE proteins (Han et al., 2004; Montecucco et al., 2005).

The work by Mohrmann et al. (2010) not only shows that the minimal number of SNAREs required for fusion is surprisingly low, but it also shows that the number of involved SNARE complexes may vary depending on the biological requirements. This helps us to explain a long-standing problem: why there is such an enormous difference in the concentration of SNAREs in trafficking organelles. For instance, the ~40 nm synaptic vesicles and the ~50 nm fusion sites at the plasma membrane contain 70–75 copies of the fusion SNAREs, respectively (discussed in van den Bogaart et al., 2010). In contrast, the density of the SNAREs functioning in the fusion of early endosomes is about 100-fold lower, although early endosomes undergo frequent and efficient fusion. Moreover, even downregulation of 90% of these SNAREs has no notable effect on the endosomal fusion (Bethani et al., 2009). The work by Mohrmann et al. (2010) indicates that this large variation in SNARE densities could be simply explained by the different needs of various intracellular membrane fusion pathways. Evoked exocytotic release of synaptic vesicles must be fast and tightly regulated, whereas for endosomal fusion, speed is less crucial and this fusion is not triggered by an action potential as in evoked release. Recent molecular dynamics simulations explain how multiple SNAREs make membrane fusion faster than a single SNARE complex (Risselada et al., 2011). In these simulations, it was found that although a single SNARE complex can catalyze membrane fusion, multiple SNARE complexes make fusion much more efficient because they facilitate the fusion pore by reducing the stability of the hemifusion intermediate. In addition, multiple SNARE complexes may also be required for the tight regulation of fusion, for instance when they need to interact with regulatory factors such as the Ca^{2+}-trigger synaptotagmin, Munc18, or complexin (Jahn and Scheller, 2006). Thus, multiple SNARE complexes might not only be just required for Ca^{2+}-triggered membrane fusion *per se*, but also for the correct recruitment, docking, and priming of the vesicles.

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