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VIEWPOINT

Roles for the SNAP25 linker domain in the fusion pore and a dynamic plasma membrane SNARE "acceptor" complex

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Central to the exocytotic release of hormones and neurotransmitters is the interaction of four SNARE motifs in proteins on the secretory granule/synaptic vesicle membrane (synaptobrevin/VAMP, v-SNARE) and on the plasma membrane (syntaxin and SNAP25, t-SNAREs). The interaction is thought to bring the opposing membranes together to enable fusion. An underlying motivation for this Viewpoint is to synthesize from recent diverse studies possible new insights about these events. We focus on a recent paper that demonstrates the importance of the linker region joining the two SNARE motifs of the neuronal t-SNARE SNAP25 for maintaining rates of secretion with roles for distinct segments in speeding fusion pore expansion. Remarkably, lipid-perturbing agents rescue a palmitoylation-deficient mutant whose phenotype includes slow fusion pore expansion, suggesting that protein–protein interactions have a role not only in bringing together the granule or vesicle membrane with the plasma membrane but also in orchestrating protein–lipid interactions leading to the fusion reaction. Unexpectedly, biochemical investigations demonstrate the importance of the C-terminal domain of the linker in the formation of the plasma membrane t-SNARE "acceptor" complex for synaptobrevin2. This insight, together with biophysical and optical studies from other laboratories, suggests that the plasma membrane SNARE acceptor complex between SNAP25 and syntaxin and the subsequent trans-SNARE complex with the v-SNARE synaptobrevin form within 100 ms before fusion.

Introduction

Most of the key proteins that regulate and initiate Ca^{2+}-dependent exocytosis have been identified, and for many their structures and interactions in vitro have been painstakingly investigated. Nevertheless, the detailed molecular interactions and structural changes of proteins and lipids that occur as individual fusion events unfold have been difficult to study in situ and are still poorly understood. An underlying motivation for this Viewpoint is to synthesize from recent diverse studies possible new insights about these events. It focuses on the underappreciated functions of the SNAP25 linker in exocytosis.

Central to the exocytotic release of hormones and neurotransmitters is the interaction of four SNARE motifs in proteins on the secretory granule/synaptic vesicle membrane (synaptobrevin/VAMP, v-SNARE) and on the plasma membrane (syntaxin and SNAP25, t-SNAREs). The interaction is thought to bring the opposing membranes together to enable fusion (Fig. 1). The trigger for fusion is the binding of Ca^{2+} to synaptotagmin on the granule membrane. Whereas synaptobrevin and syntaxin each contain one SNARE motif, SNAP25 contains two SNARE motifs (SN1 and SN2) that are joined by a palmitoylated linker in the t-SNARE, SNAP25 (Fig. 2). The four SNARE motifs form a stable four-helical bundle complex in vitro (Sutton et al., 1998) that probably reflects a structure that forms after fusion when the proteins are in the same membrane (cis conformation).

It has been known for some time that palmitoylation enables binding of SNAP25 to the plasma membrane (Gonzalo et al., 1999). Additional functions for the linker have also been discovered. First, joining SN1 and SN2 together with a palmitoylated linker facilitates the formation of membrane complexes with syntaxin that bring the N termini of SN1 and SN2 close together, as reflected by increased FRET in labeled probes (Fig. 3, A and B; Wang et al., 2008). Second, the SNAP25 linker enables fast exocytosis as revealed in two types of experiments. Separating SN1 and SN2 reduces robust secretion in biochemical experiments (Wang et al., 2008). Modifications of the linker decrease fusion rates in high temporal resolution electrophysiological experiments (Nagy et al., 2008).

The SNAP25 linker and fusion pore expansion

A recent important study by Shaaban et al. (2019) using single cell, high temporal resolution electrophysiological recordings

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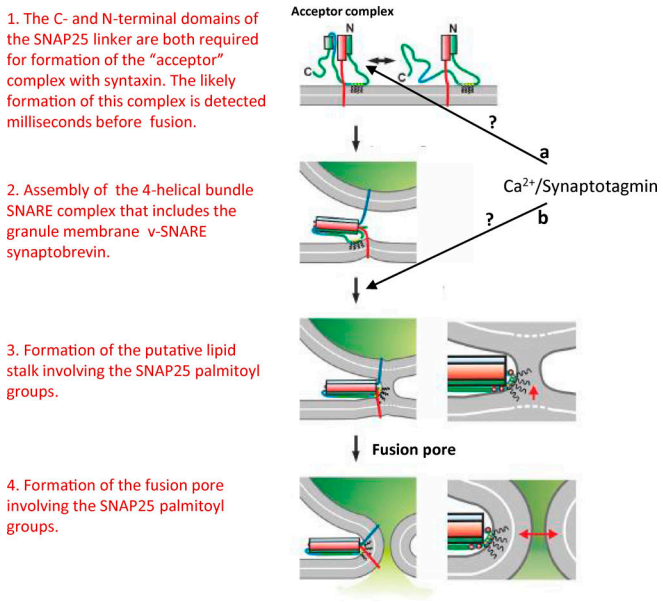

Figure 1. **Late steps in the fusion pathway.** This figure is an adaptation of Figure 9 from Shaaban et al. (2019). It adds the possibility that Ca^{2+}/synaptotagmin promotes formation of the acceptor complex as well as the fusion pore. Figure 1 is reprinted with permission from *eLife*.

confirms the importance of the linked SNARE domains in exocytosis and greatly expands our understanding of linker function. SNAP25 analogues differing only in the linker were introduced by viral infection into chromaffin cells from SNAP25 knockout mice.

Full substitution of the linker domain with a flexible peptide produced a nonfunctional SNAP25 mutant. A careful structure-function investigation demonstrated important and different functions for the N-terminal and C-terminal domains of the linker. Compared with wild-type SNAP25, partial substitution of segments at either end of the linker greatly reduced the amount of primed secretion and slowed the rate of fusion. The N terminus of the linker immediately adjacent to SN1 has four cysteine residues, which are sites for palmitoylation (Fig. 2). In this study and in an earlier study (Nagy et al., 2008), amperometry demonstrated that cysteine-to-serine mutations greatly prolonged the initial fusion pore expansion as measured by the prespike foot, *without significantly altering the main catecholamine release spike*. This is a beautiful result indicating that palmitoylation not only causes plasma membrane binding but also specifically speeds expansion of the fusion pore. The location of the palmitoylation sites is also critical since fusion pore expansion is greatly slowed in mutants in which the palmitoylation sites were relocated away from the wild-type position. The authors postulated that if the membrane interactions of the N-terminal domain of the linker are important for lipid intermediates in fusion, then membrane-active agents might rescue the effects of a N-terminal segment mutant without the palmitoylation sites. Remarkably, intracellular application of the membrane-active reagents methanol and oleic acid reduced or reversed the effects of the mutant protein. The authors suggest that the linker-mediated lipid interaction directly regulates membrane curvature at the initial fusion pore, thereby facilitating fusion pore expansion.

The SNAP25 linker and the formation of the plasma membrane prefusion acceptor complex, a possible late step that helps initiate fusion

The C-terminal region of the linker adjacent to SN2 (in addition to the N-terminal linker palmitoylation domain adjacent to SN1) is necessary for rapid exocytosis (Nagy et al., 2008; Shaaban et al., 2019). Importantly, there are compelling biochemical and structural data that support a role of this region in fusion. The C-terminal linker segment (Fig. 2, yellow line) strongly facilitates the in vitro binding of SN2 to the readily formed complexes of SN1 and syntaxin (Shaaban et al., 2019). It was independently found that the linker–membrane interaction increases linker helical content, which enables the C-terminal linker region to interact with syntaxin (Jiang et al., 2019). These are critical findings. They explain the ability of membrane-bound syntaxin to bring the N termini of linked, but not separated, SN1 and SN2 into close proximity, as detected with an intramolecular FRET construct of SNAP25 (Fig. 3, A and B; Wang et al., 2008). This conformation of the t-SNARE "acceptor" complex likely initiates interaction with the v-SNARE, synaptobrevin2. In an experimental tour de force, it was found using a similar intramolecular SNAP25 FRET construct that FRET increased on average 100 ms before fusion at fusion sites in chromaffin cells (Fig. 3 C; Zhao et al., 2013). Since the in vitro experiments indicate that the FRET increase reflects formation of the acceptor complex, the various *experiments suggest that the acceptor complex is dynamic and its formation is an important late step required to initiate fusion.* This concept is consistent with the finding that granules continue to jitter as much as a 100 nm (much greater than the ∼10 nm length of the tetrahelical SNARE complex; Hanson et al., 1997) within 100 ms of fusion (Allersma et al., 2006; Degtyar et al., 2007) and suggests that trans-SNARE complexes form just milliseconds before fusion (Degtyar et al., 2007; Jahn and Fasshauer, 2012). The key biochemical trigger of

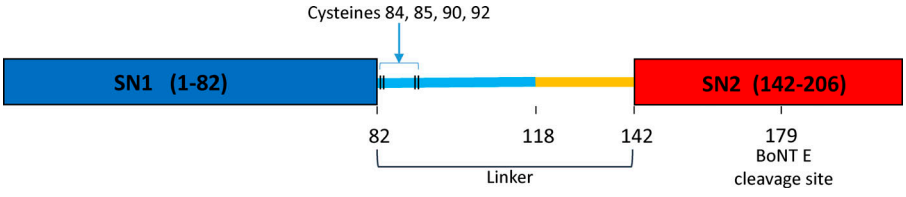

Figure 2. **Schematic of the SNAP25.** SNAP25 contributes two of the four SNARE motifs that comprise the prototypical four-helical bundle in neuronal SNARE proteins. The two motifs, SN1 and SN2, are joined by a linker sequence (light blue and yellow line) with four N-terminal cysteines that are sites for palmitoylation. The linker region is divided into N-terminal (blue) and C-terminal (yellow) domains according to the constructs in Shaaban et al. (2019). SN2 contains the cleavage site for botulinum neurotoxin type E (BoNT E). BoNT E was used in an earlier study to inactivate endogenous SNAP25 (Wang et al., 2008). Shaaban et al., (2019) demonstrate that the N- and C-terminal domains have different roles in enabling the function of SNAP25 in exocytosis. The horizontal distances are to scale.

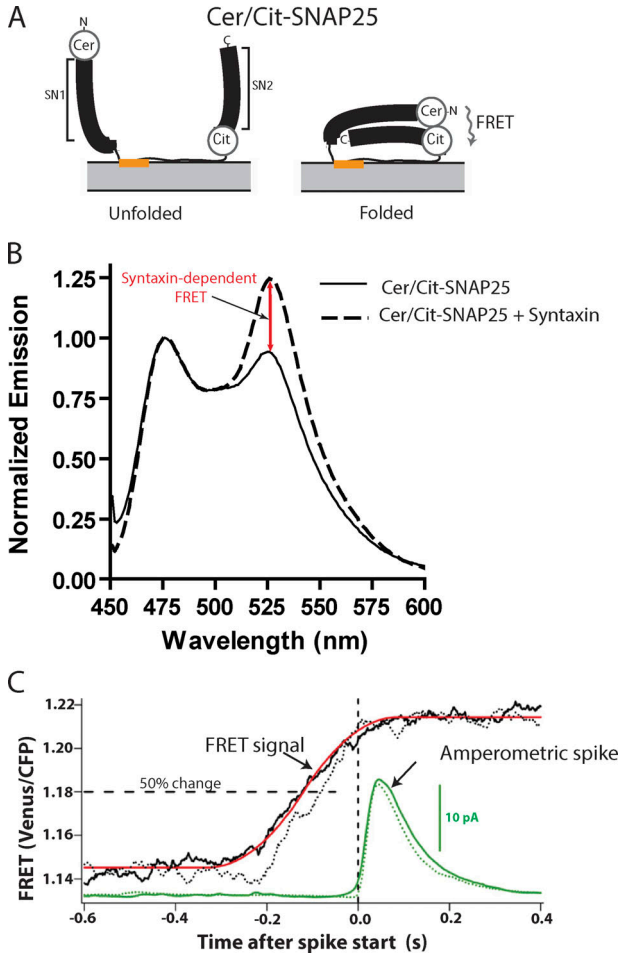

Figure 3. **A SNAP25 intramolecular FRET probe signals the formation of the acceptor complex in the plasma membrane ~100 ms before fusion. (A)** Rationale of the use of the SNAP25 FRET probe to examine structural changes in SNAP25 upon interaction with syntaxin. Cerulean (Cer) and citrine (Cit) were used as the donor and acceptor fluorophores in the experiments of Wang et al. (2008). The orange rectangle in the N-terminal domain of the linker reflects palmitoylated cysteines. CFP and Venus were used as the donor and acceptor fluorophores in the experiments of Zhao et al. (2013). **(B)** The SNAP25 FRET probe detects interaction with syntaxin in biological membranes (Wang et al., 2008). HEK293T cells were transfected with a plasmid encoding the SNAP-25 FRET probe alone or together with a plasmid encoding syntaxin. Shown are the emission spectra of a suspended membrane fraction excited at 435 nm. The increased emission at 526 nm upon coexpression of syntaxin reflects a conformational change that brings the N termini of SN1 and SN2 closer together. From Wang et al. (2008). **(C)** FRET increases at the fusion site ~100 ms before fusion pore formation (Zhao et al., 2013). Chromaffin cells were infected with virus to express the SNAP25 FRET probe. The time and location of fusion events in single cells were detected using a four-electrode electrochemical array to measure catecholamine release from individual granules. Total internal reflection fluorescence microscopy was used to detect local FRET changes. FRET changes from many hundreds of events were aligned to the beginning of the amperometric spike. A method was devised to account for the random timing of shutter opening relative to the fusion event. The timing of the half-maximal FRET change (dashed horizontal line) reflects the average time of the FRET change relative to fusion pore formation. It occurred 90 ms before amperometric spikes without a prespike foot, and 120 ms before amperometric spikes with a prespike foot. Fig. 3 is reprinted with permission from *Proc. Natl. Acad. Sci. USA*.

the fusion event then would occur upstream of trans-SNARE complex formation, as has been postulated (Jahn and Fasshauer, 2012). Complete N- to C-terminal zippering (Gao et al., 2012) would have to start and finish within 100 ms of fusion.

The concept of a "primed" granule pool to account for fast fusion events upon a Ca^{2+} stimulus is firmly imbedded in kinetic models of exocytosis (Voets, 2000). The above considerations make it unlikely that the primed pool represents granules with stable, partially zippered trans-SNARE complexes (Hua and Charlton, 1999; Xu et al., 1999; Fasshauer and Margittai, 2004; Walter et al., 2010). Instead, the primed pool may reflect a subset of granules closely apposed to the plasma membrane that are properly engaged with the numerous protein regulators (i.e., complexin, nSEC1, and Munc13) and lipid regulators (i.e., plasma membrane PI-4,5-P_2 [phosphatidylinositol 4,5-bisphosphate]) of fusion. If this is correct, then Ca^{2+} and synaptotagmin may trigger fusion in part by coupling to and perhaps catalyzing the formation of the plasma membrane SNAP25/syntaxin acceptor complex (Fig. 1, arrow a), thereby enabling rapid trans-SNARE complex formation (Fig. 1, step 2) and fusion (Fig. 1, steps 3 and 4). This notion contrasts with a model in which Ca^{2+}-bound synaptotagmin acts to trigger fusion *only after* formation of a stable, partially zippered trans-SNARE complex (Fig. 1, arrow b; Südhof, 2013).

Protein–lipid interactions in exocytosis

The specific, position-dependent effect on fusion pore expansion of palmitoyl groups in the linker domain in SNAP25 highlights the importance of protein–lipid as well as protein–protein interactions in the late steps of exocytosis. Numerous other protein–lipid interactions have been elucidated among components of the fusion complex. The first evidence for a specific role of lipids in Ca^{2+}-dependent exocytosis was the discovery of the requirement for the highly negatively charged plasma membrane phospholipid PI-4,5-P_2 (Eberhard et al., 1990; Hay and Martin, 1993; Hay et al., 1995; Holz et al., 2000; Milosevic et al., 2005). Synaptotagmin, the Ca^{2+} sensor for exocytosis on the secretory granule membrane, interacts through electrostatic binding to plasma membrane PI-4,5-P_2 (Bai et al., 2004). This interaction is thought to bring the granule and plasma membrane into close apposition, allowing subsequent interaction with plasma membrane phosphatidyl serine to trigger fusion (Zhang et al., 2010). A lipid-binding domain in syntaxin1A that binds negatively charged lipids and cellular phospholipase D (which produces the negatively charged phosphatidic acid) are both necessary for normal fusion pore dynamics (Lam et al., 2008). Flexibility in the transmembrane domain of synaptobrevin enhances the fusion reaction and, importantly, also speeds fusion pore expansion (Dhara et al., 2016). Although there has been considerable progress in understanding the structural basis of protein–protein interactions leading to formation of the core fusion complex, we have little understanding how these protein–protein interactions orchestrate the above protein–lipid interactions necessary for normal exocytosis.

Summary

The domain in SNAP25 that links its two SNARE motifs can no longer be considered a minor player in exocytosis as had been

suggested by earlier work (Chen et al., 1999). This is highlighted in the important study by Shaaban et al. (2019). The linker participates in the formation of the t-SNARE receptor complex with syntaxin (Jiang et al., 2019; Shaaban et al., 2019) is required for robust secretion (Wang et al., 2008; Shaaban et al., 2019) and functions to quicken fusion pore expansion and to control secretion rates (Shaaban et al., 2019). Normal function requires precise localization of the palmitoylated residues in the linker. Furthermore, biochemical investigations demonstrate the importance of the C-terminal domain of the linker in the formation of the plasma membrane t-SNARE acceptor complex for synaptobrevin2 (Jiang et al., 2019; Shaaban et al., 2019). This insight, together with in vitro and in situ FRET measurements of fluorescent SNAP25 (Wang et al., 2008; Zhao et al., 2013), raises the possibility that the plasma membrane SNARE acceptor complex forms ∼100 ms before fusion, thereby enabling engagement with granule membrane synaptobrevin2 and other granule membrane proteins to catalyze fusion.

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References

Allersma, M.W., M.A. Bittner, D. Axelrod, and R.W. Holz. 2006. Motion matters: secretory granule motion adjacent to the plasma membrane and exocytosis. *Mol. Biol. Cell*. 17:2424–2438. https://doi.org/10.1091/mbc.e05-10-0938

Bai, J., W.C. Tucker, and E.R. Chapman. 2004. PIP2 increases the speed of response of synaptotagmin and steers its membrane-penetration activity toward the plasma membrane. *Nat. Struct. Mol. Biol*. 11:36–44. https://doi.org/10.1038/nsmb709

Chen, Y.A., S.J. Scales, S.M. Patel, Y.C. Doung, and R.H. Scheller. 1999. SNARE complex formation is triggered by Ca2+ and drives membrane fusion. *Cell*. 97:165–174. https://doi.org/10.1016/S0092-8674(00)80727-8

Degtyar, V.E., M.W. Allersma, D. Axelrod, and R.W. Holz. 2007. Increased motion and travel, rather than stable docking, characterize the last moments before secretory granule fusion. *Proc. Natl. Acad. Sci. USA*. 104:15929–15934. https://doi.org/10.1073/pnas.0705406104

Dhara, M., A. Yarzagaray, M. Makke, B. Schindeldecker, Y. Schwarz, A. Shaaban, S. Sharma, R.A. Böckmann, M. Lindau, R. Mohrmann, et al. 2016. v-SNARE transmembrane domains function as catalysts for vesicle fusion. *eLife*. 5. e17571. https://doi.org/10.7554/eLife.17571

Eberhard, D.A., C.L. Cooper, M.G. Low, and R.W. Holz. 1990. Evidence that the inositol phospholipids are necessary for exocytosis. Loss of inositol phospholipids and inhibition of secretion in permeabilized cells caused by a bacterial phospholipase C and removal of ATP. *Biochem. J*. 268:15–25. https://doi.org/10.1042/bj2680015

Fasshauer, D., and M. Margittai. 2004. A transient N-terminal interaction of SNAP-25 and syntaxin nucleates SNARE assembly. *J. Biol. Chem*. 279:7613–7621. https://doi.org/10.1074/jbc.M312064200

Gao, Y., S. Zorman, G. Gundersen, Z. Xi, L. Ma, G. Sirinakis, J.E. Rothman, and Y. Zhang. 2012. Single reconstituted neuronal SNARE complexes zipper in three distinct stages. *Science*. 337:1340–1343. https://doi.org/10.1126/science.1224492

Gonzalo, S., W.K. Greentree, and M.E. Linder. 1999. SNAP-25 is targeted to the plasma membrane through a novel membrane-binding domain. *J. Biol. Chem*. 274:21313–21318. https://doi.org/10.1074/jbc.274.30.21313

Hanson, P.I., R. Roth, H. Morisaki, R. Jahn, and J.E. Heuser. 1997. Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell*. 90:523–535. https://doi.org/10.1016/S0092-8674(00)80512-7

Hay, J.C., and T.F. Martin. 1993. Phosphatidylinositol transfer protein required for ATP-dependent priming of Ca(2+)-activated secretion. *Nature*. 366:572–575. https://doi.org/10.1038/366572a0

Hay, J.C., P.L. Fisette, G.H. Jenkins, K. Fukami, T. Takenawa, R.A. Anderson, and T.F. Martin. 1995. ATP-dependent inositide phosphorylation required for Ca(2+)-activated secretion. *Nature*. 374:173–177. https://doi.org/10.1038/374173a0

Holz, R.W., M.D. Hlubek, S.D. Sorensen, S.K. Fisher, T. Balla, S. Ozaki, G.D. Prestwich, E.L. Stuenkel, and M.A. Bittner. 2000. A pleckstrin homology domain specific for phosphatidylinositol 4, 5-bisphosphate (PtdIns-4,5-P2) and fused to green fluorescent protein identifies plasma membrane PtdIns-4,5-P2 as being important in exocytosis. *J. Biol. Chem*. 275:17878–17885. https://doi.org/10.1074/jbc.M000925200

Hua, S.Y., and M.P. Charlton. 1999. Activity-dependent changes in partial VAMP complexes during neurotransmitter release. *Nat. Neurosci*. 2:1078–1083. https://doi.org/10.1038/16005

Jahn, R., and D. Fasshauer. 2012. Molecular machines governing exocytosis of synaptic vesicles. *Nature*. 490:201–207. https://doi.org/10.1038/nature11320

Jiang, X., Z. Zhang, K. Cheng, Q. Wu, L. Jiang, G.J. Pielak, M. Liu, and C. Li. 2019. Membrane-mediated disorder-to-order transition of SNAP25 flexible linker facilitates its interaction with syntaxin-1 and SNARE-complex assembly. *FASEB J*. 33:7985–7994. https://doi.org/10.1096/fj.201802796R

Lam, A.D., P. Tryoen-Toth, B. Tsai, N. Vitale, and E.L. Stuenkel. 2008. SNARE-catalyzed fusion events are regulated by Syntaxin1A-lipid interactions. *Mol. Biol. Cell*. 19:485–497. https://doi.org/10.1091/mbc.e07-02-0148

Milosevic, I., J.B. Sørensen, T. Lang, M. Krauss, G. Nagy, V. Haucke, R. Jahn, and E. Neher. 2005. Plasmalemmal phosphatidylinositol-4,5-bisphosphate level regulates the releasable vesicle pool size in chromaffin cells. *J. Neurosci*. 25:2557–2565. https://doi.org/10.1523/JNEUROSCI.3761-04.2005

Nagy, G., I. Milosevic, R. Mohrmann, K. Wiederhold, A.M. Walter, and J.B. Sørensen. 2008. The SNAP-25 linker as an adaptation toward fast exocytosis. *Mol. Biol. Cell*. 19:3769–3781. https://doi.org/10.1091/mbc.e07-12-1218

Shaaban, A., M. Dhara, W. Frisch, A. Harb, A.H. Shaib, U. Becherer, D. Bruns, and R. Mohrmann. 2019. The SNAP-25 linker supports fusion intermediates by local lipid interactions. *eLife*. 8. e41720. https://doi.org/10.7554/eLife.41720

Südhof, T.C.. 2013. Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. *Neuron*. 80:675–690. https://doi.org/10.1016/j.neuron.2013.10.022

Sutton, R.B., D. Fasshauer, R. Jahn, and A.T. Brunger. 1998. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature*. 395:347–353. https://doi.org/10.1038/26412

Voets, T.. 2000. Dissection of three Ca2+-dependent steps leading to secretion in chromaffin cells from mouse adrenal slices. *Neuron*. 28:537–545. https://doi.org/10.1016/S0896-6273(00)00131-8

Walter, A.M., K. Wiederhold, D. Bruns, D. Fasshauer, and J.B. Sørensen. 2010. Synaptobrevin N-terminally bound to syntaxin-SNAP-25 defines the primed vesicle state in regulated exocytosis. *J. Cell Biol*. 188:401–413. https://doi.org/10.1083/jcb.200907018

Wang, L., M.A. Bittner, D. Axelrod, and R.W. Holz. 2008. The structural and functional implications of linked SNARE motifs in SNAP25. *Mol. Biol. Cell*. 19:3944–3955. https://doi.org/10.1091/mbc.e08-04-0344

Xu, T., B. Rammner, M. Margittai, A.R. Artalejo, E. Neher, and R. Jahn. 1999. Inhibition of SNARE complex assembly differentially affects kinetic components of exocytosis. *Cell*. 99:713–722. https://doi.org/10.1016/S0092-8674(00)81669-4

Zhang, Z., E. Hui, E.R. Chapman, and M.B. Jackson. 2010. Regulation of exocytosis and fusion pores by synaptotagmin-effector interactions. *Mol. Biol. Cell*. 21:2821–2831. https://doi.org/10.1091/mbc.e10-04-0285

Zhao, Y., Q. Fang, A.D. Herbst, K.N. Berberian, W. Almers, and M. Lindau. 2013. Rapid structural change in synaptosomal-associated protein 25 (SNAP25) precedes the fusion of single vesicles with the plasma membrane in live chromaffin cells. *Proc. Natl. Acad. Sci. USA*. 110:14249–14254. https://doi.org/10.1073/pnas.1306699110