Models of synaptotagmin-1 to trigger Ca2+-dependent vesicle fusion

Park, Yongsoo; Ryu, Je Kyung

DOI
10.1002/1873-3468.13193

Publication date
2018

Document Version
Final published version

Published in
FEBS Letters

FEBS Letters

FEBS PRESS science publishing by scientists

REVIEW ARTICLE

Models of synaptotagmin-1 to trigger Ca^{2+}-dependent vesicle fusion

Yongsoo Park[1] and Je-Kyung Ryu[2]

1 Department of Molecular Biology and Genetics, Koç University, Istanbul, Turkey
2 Department of Bionanoscience, Kavli Institute of Nanoscience Delft, Delft University of Technology, The Netherlands

Correspondence
Y. Park, Department of Molecular Biology and Genetics, Koç University, Istanbul 34450, Turkey
Fax: +90 212 338 1559
Tel: +90 212 338 1815
E-mail: ypark@ku.edu.tr
and
J.-K. Ryu, Department of Bionanoscience, Kavli Institute of Nanoscience Delft, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands
Fax: +31-(0)15-2781202
Tel: +31-(0)15-2788756
E-mail: j.ryu@tudelft.nl

(Received 3 May 2018, revised 2 July 2018, accepted 6 July 2018, available online 30 July 2018)

doi:10.1002/1873-3468.13193

Edited by Wilhelm Just

Vesicles in neurons and neuroendocrine cells store neurotransmitters and peptide hormones, which are released by vesicle fusion in response to Ca^{2+}-evoking stimuli. Synaptotagmin-1 (Syt1), a Ca^{2+} sensor, mediates ultrafast exocytosis in neurons and neuroendocrine cells. After vesicle docking, Syt1 has two main groups of binding partners: anionic phospholipids and the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) complex. The molecular mechanisms by which Syt1 triggers vesicle fusion remain controversial. This Review introduces and summarizes six molecular models of Syt1: (a) Syt1 triggers SNARE unclamping by displacing complexin, (b) Syt1 clamps SNARE zippering, (c) Syt1 causes membrane curvature, (d) membrane bridging by Syt1, (e) Syt1 is a vesicle-plasma membrane distance regulator, and (f) Syt1 undergoes circular oligomerization. We discuss important conditions to test Syt1 activity *in vitro* and attempt to illustrate the possible roles of Syt1.

Keywords: complexin; neurotransmitter; peptide hormone; SNARE; vesicle fusion; synaptotagmin-1

Neurons and neuroendocrine cells communicate by exocytosis of neurotransmitters at chemical synapses. Neurotransmitters are packaged into specialized organelles called vesicles, i.e., synaptic vesicles and dense core-vesicles. Synaptic vesicles from the presynapse transfer classical neurotransmitters (e.g., glutamate, acetylcholine, gamma-aminobutyric acid, glycine) that regulate electrical signals to postsynapse, whereas dense core-vesicles are responsible for exocytosis of amines, neuropeptides, and hormones, which modulate synaptic activity [1–3].

Dense core-vesicles are minor components (e.g., 1 ~ 2% of density of synaptic vesicles) in the central nervous system such as the neuromuscular junction [4], thalamus [5], cerebellum [5], and hippocampus [6]. For this reason, chromaffin granules called large dense-core vesicles (LDCVs) have been widely used as a model system to study dense core-vesicles [7]. Despite the differences in the biological functions of synaptic vesicles and LDCVs [2], both types use soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins as the fusion machinery [1–3,8]. Ca^{2+} sensor synaptotagmin-1 (Syt1), which is anchored in vesicle membranes, mediates fast Ca^{2+}-dependent exocytosis of synaptic vesicles in neuromuscular junctions [9] and hippocampal neurons [10], and of LDCVs in chromaffin cells [11,12].

Abbreviations
LDCV, large dense-core vesicle; PI, phosphatidylinositol; PKC, protein kinase C; PS, phosphatidylserine; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors; Syt1, synaptotagmin-1.

Evidence in 1990 showed that the C2 domains of Syt1 have membrane-binding affinity and suggested Syt1 as the Ca^{2+} sensor [13], but further reports have suggested several different molecular mechanisms of how Ca^{2+} triggers Syt1 to initiate vesicle fusion, so the topic has become increasingly enigmatic and even controversial.

This Review focuses on six molecular models to explain the function of Syt1 in Ca^{2+}-dependent vesicle fusion, and discusses the complexity of Syt1 functions: (a) Syt1 triggers SNARE unclamping by displacing complexin, (b) Syt1 clamps SNARE zippering, (c) Syt1 causes membrane curvature, (d) membrane bridging by Syt1, (e) Syt1 is a vesicle-plasma membrane distance regulator, and (f) Syt1 undergoes circular oligomerization.

Syt1 as a Ca^{2+} sensor for fast exocytosis

Neurotransmitters contained in synaptic vesicles are released in response to Ca^{2+} influx at presynaptic nerve terminals [14]. Synaptic transmission at the synapses is fast: the delay from presynapse to postsynapse is around 0.2 ~ 1 ms, depending on temperature; at 37 °C, vesicle fusion occurs within 10 ~ 100 μs after Ca^{2+} influx in the presynapse [15,16]. Syt1 is responsible for fast synchronous exocytosis of synaptic vesicles and LDCV [17,18], but not for asynchronous release, i.e., other Ca^{2+} sensors are involved in asynchronous release (reviewed in [17]). Syt1 that contains two tandem C2 regions, homologous to protein kinase C (PKC) [13] and proposed to be a Ca^{2+} sensor [19], interacts with negatively charged anionic phospholipids in a Ca^{2+}-dependent manner. Knockout of Syt1 in hippocampal neurons causes selective impairment of fast synchronous exocytosis [10]; this result supports the direct evidence that Syt1 is a Ca^{2+} sensor for synchronous release. Mammalian Syt includes 16 isoforms [20], but Syt1, 2, and 9 mediate Ca^{2+}-dependent fusion of synaptic vesicles [21] in neurons, whereas Syt1 and 7 cause Ca^{2+}-dependent LDCV exocytosis [22] in chromaffin cells.

Syt1 is a transmembrane protein in synaptic vesicles and LDCVs. Acidic aspartate residues in two Ca^{2+}-binding C2 domains [13] (C2A and C2B), coordinate three and two Ca^{2+} ions, respectively [23–25]. In addition to these Ca^{2+}-binding loops, the C2B domain contains a polybasic region (KKKK, 324–327; Fig. 1) that is close to Ca^{2+}-binding loops and is enriched with lysine residues [23] that interact with anionic phospholipids [26–32] or the SNARE complex [33–44]. Evidence in 1992 showed that Syt1 interacts with anionic membrane [19] or with SNARE proteins [39] by electrostatic interaction. Whether Syt1 can interact with anionic phospholipids or the SNARE complex, or both in a physiological ionic environment remains controversial.

Molecular mechanisms of Syt1 to trigger fusion

Model 1: SNARE unclamping by displacing complexin

Complexin I and II are considered to bind to the partially zippered SNARE complex and might block progression of SNARE zippering by replacing synaptobrevin/VAMP-2 binding in the C-terminal part of the SNARE complex [45–48] (Model 1, Fig. 2). However, the hypothesis that complexin clamps SNARE zippering might be applicable only in a specialized synapse and this 'SNARE clamping by complexin' hypothesis remains controversial [49], e.g., complexin might promote progression of SNARE zippering [50], complexin might inhibit spontaneous fusion throughout the interaction of membrane [51] and t-SNARE [52], and complexin may clamp vesicle fusion by electrostatic repulsion (reviewed in ref. [49]). Complexin I might block vesicle fusion by the electrostatic repulsion between the vesicle membrane and complexin I in mouse hippocampal neurons [53], but electrostatic repulsion by complexin I showed no significant effect in *C. elegans* neuromuscular junction [54]. The phenotype of complexin I knockout varies among species, so generalization of complexin function is a complicated task. Despite this debate, complexins are generally accepted to be clamping factors that block vesicle fusion in a docking state [45,46,55]. Upon Ca^{2+} influx, Syt1 interacts with the partially zippered SNARE complex and displaces complexin from SNAREs, so the clamping effect of complexin might be released by Ca^{2+}-bound Syt1.

Many groups have demonstrated Syt1 interaction with SNARE proteins [33–44]. The polybasic region in the C2B domain of Syt1 (KKKK, 324–327; Fig. 1) is associated with SNAREs [36–38,56,57]. NMR structure suggested that the polybasic region of the C2B domain (KKKK, 324–327; Fig. 1) interacts electrostatically with acidic residues from syntaxin-1 and SNAP-25 of the SNARE complex, whereas basic residues from Syt1 (bottom end, RR, 398 399; Fig. 1) binds to the membrane [57]; NMR and molecular dynamics simulation suggest that Syt1 and complexin I interact simultaneously with a partially assembled SNARE complex that has distinct binding sites [57]. In contrast, atomic-resolution X-ray crystal structures

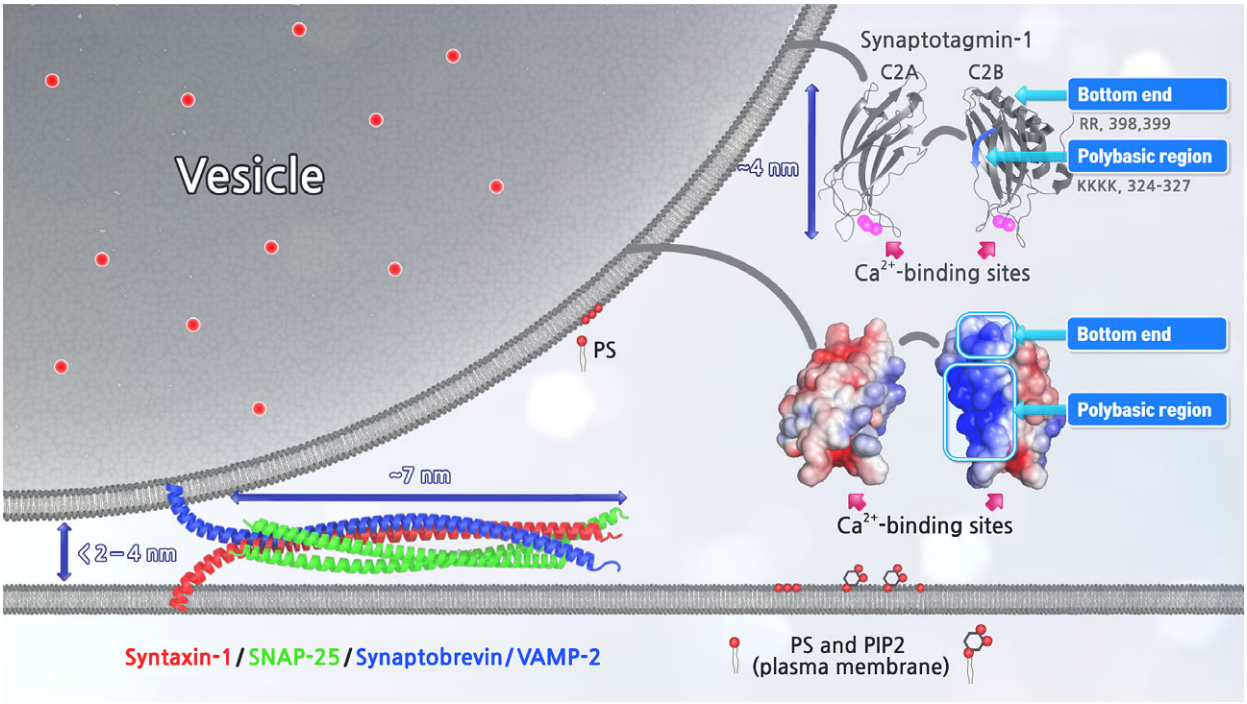

Fig. 1. Structure of the SNARE complex and Syt1. The SNARE motif of the SNARE complex is ~ 7 nm long and synaptic vesicles are tightly docked < 2 ~ 4 nm from the plasma membrane; this proximity suggests that SNARE proteins might be partially assembled in a docking state. The C2B domain of Syt1 has a polybasic region with a four-lysine patch (KKKK, 324–327) and bottom end with two arginines (RR, 398 399), which are responsible for the interaction with SNAREs or PIP_2. Protein structures (top) and the electrostatic potential surface (bottom) are visualized using PyMOL Molecular Graphics System (PDB ID: 1BYN for C2A, 1K5W for C2B, and 1SFC for the SNARE complex).

showed that the polybasic region of the C2B domain is not involved in SNARE interaction, but binds to the plasma membrane [58]; and that instead, three different Syt1–SNARE binding sites exist, i.e., the C2A domain of the tertiary interface bridges another SNARE complex and could displace complexin from the SNARE complex [58]. The conserved primary interface involves acidic residues from SNAP-25 and syntaxin-1A and basic residues from Syt1 (bottom end, RR, 398 399; Fig. 1) [58], but an NMR-based model suggests that this region of Syt1 (bottom end, RR, 398 399; Fig. 1) binds to the plasma membrane [57]. These diverse results all indicate that the electrostatic effect dominantly mediates the interaction of Syt1 and the SNARE complex, and that this interaction depends on ionic strength [43,48].

Recent high-resolution crystal structures revealed an additional primed SNARE–Complexin–Syt1 tripartite interface in which two Syt1 molecules simultaneously interact with one SNARE complex on distinct binding sites on primary and tripartite interfaces, termed Syt1–SNARE–Complexin–Syt1 structures [59]. Syt1–SNARE–Complexin–Syt1 complexes might keep the partially zippered SNARE complex in a vesicle-priming state [59]. Ca^{2+}-bound Syt1 causes molecular rearrangements, and ultimately induces full SNARE zippering by liberating or displacing complexin from the Syt1–SNARE–Complexin–Syt1 complex [59].

However, this model in which Syt1 leads to unclamping of SNARE zippering by displacing complexin is still debated. Syt1 binds to SNAP-25 [60–62] and syntaxin-1A [42,62,63] and this interaction is mediated by the electrostatic effect between basic residues of C2AB domain and acidic residues of syntaxin-1A [64] or SNAP-25 [61,65]. Electrostatic interaction between Syt1 and the SNARE complex is mainly observable at low ionic strength [43,48], because under this condition the weak electrostatic interaction strengthens. This interaction of Syt1–SNARE has very weak affinity [31] and is mainly observable at very high concentrations (10 ~ 400 μM) of the C2AB domain of Syt1 [42,60,64–66] and in buffer that has low ionic strength (50 ~ 100 mM Na^+ or K^+) [43,48,67]. Complexin has a high binding affinity, so it can interact with the SNARE complex independently of ionic strength, but Syt1 fails to bind to the SNARE complex even in the presence of complexin at physiological ionic strength [67].

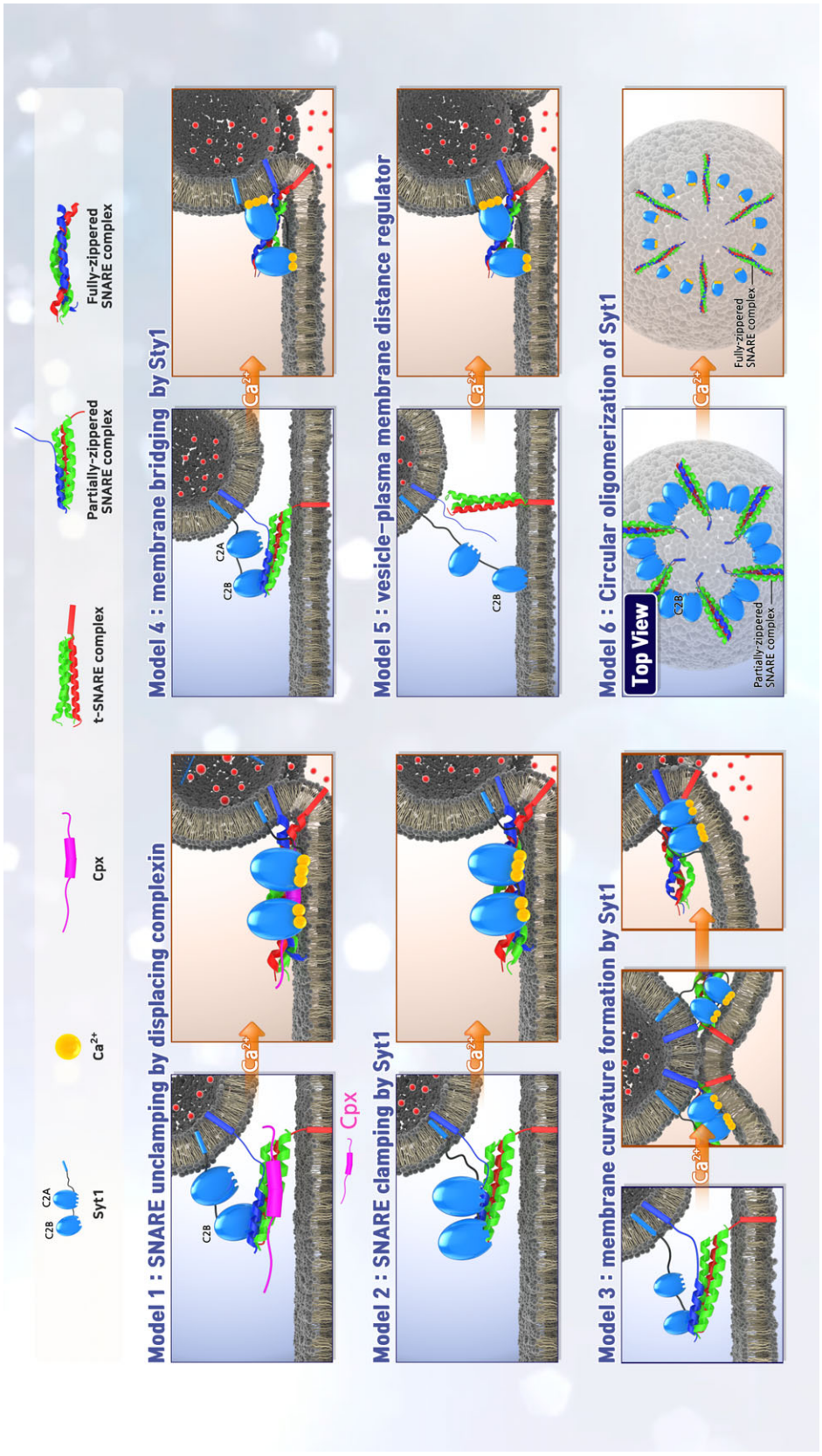

Fig. 2. Six molecular models of Syt1. (Model 1) Syt1-mediated SNARE unclamping by displacing complexin. Complexin clamps the full SNARE zippering before Ca^{2+} arrival. Ca^{2+}–bound Syt1 displaces the complexin so that the partially zippered SNARE complex becomes fully zippered. (Model 2) Syt1-mediated SNARE clamping. Syt1 itself clamps the partially zippered SNARE complex. Ca^{2+}–bound Syt1 is released from clamping the SNARE complex and inserted into the plasma membrane, thus triggering the full SNARE zippering for vesicle fusion. (Model 3) Membrane curvature formation. Ca^{2+}-bound Syt1 forms the local positive curvature in the plasma membrane, thereby lowering energy barrier for SNARE-mediated fusion. (Model 4) Membrane bridging. An equilibrium between partially zippered SNARE complex and repulsion between the two membranes. After Ca^{2+} arrival, C2A and C2B domains bind to opposing membranes to induce the full SNARE zippering and vesicle fusion. (Model 5) Vesicle-plasma membrane distance regulator. The C2B domain of Syt1 binds to the plasma membrane, and after Ca^{2+} arrival, Syt1 bridges two membranes as a 'charge bridge' to initiate the SNARE complex formation to trigger vesicle fusion. (Model 6) Circular oligomerization. A circular oligomerization of Syt1 forms on the plasma membrane by the C2B domains, and thereby blocks full SNARE zippering. Ca^{2+} binding to Syt1 dissociates the ring oligomer so that full SNARE zippering and vesicle fusion occur.

Currently, no direct evidence shows that Syt1 triggers full SNARE zippering by displacing or replacing complexin, which arrests vesicle fusion. Whether complexin clamps SNARE is still under debate, because spontaneous vesicle fusion is either unaffected or reduced in complexin-deficient mammalian neurons (reviewed in Ref. [49]); furthermore, in the physiological ionic environment Syt1 dominantly binds to anionic phospholipids without interacting with the SNARE complex [31,67]. However, complexin could inhibit spontaneous fusion by other processes, for example, the interaction of membrane–complexin [51] and t-SNARE–complexin [52].

Model 2: Syt1 clamps SNARE zippering

Another hypothesis (Model 2, Fig. 2) suggests that Syt1, not complexin, is the clamping factor of SNARE zippering. This hypothesis was proposed because deletion of Syt1 increases spontaneous release (mini-frequency) in many cell types [9,37,68–72], i.e., SNARE clamping inhibits spontaneous release before Ca^{2+} influx. The model proposes that Syt1 clamps SNARE zippering to arrest vesicle fusion before Ca^{2+} triggering, and that Ca^{2+}-bound Syt1 is inserted to the plasma membrane, and thereby causes conformational changes that evoke full SNARE zippering and vesicle fusion.

The C2AB domain of Syt1 has been suggested to be a clamping factor that halts full SNARE zippering by binding to the partially assembled SNARE complex [37]. SNARE clamping by Syt1 may steer the Ca^{2+}-triggered membrane penetration of Syt1 [37]. However, the full-length of Syt1 fails to clamp vesicle fusion, but instead slightly increases the efficiency of Ca^{2+}-independent fusion [29,73–75]; this result strongly argues that Syt1 is not a clamping factor of SNARE assembly. The inhibitory effect of the C2AB domain of Syt1 is also controversial in different systems, e.g., inhibition of Ca^{2+}-independent fusion by the C2AB domain does not occur when purified native vesicles are used [76]. Syt1 inhibits spontaneous release of vesicle fusion, but the mechanisms of SNARE clamping by Syt1 are under debate.

Furthermore, the weak Syt1–SNARE interaction is completely disrupted at physiological ion concentrations that include 150 mM K^+ and 1 mM ATP/Mg^{2+} [67]; this observation may refute the hypothesis that Syt1 clamps SNARE. However, the interaction of the C2B domain (bottom end, RR, 398 399; Fig. 1) with the SNARE complex is resistant to ionic strength and observable independently of the presence of ATP/Mg^{2+}; this result contradicts the influence of ionic strength on Syt1–SNARE interaction [77]. The electrostatic interaction is tightly coupled to the ionic strength of the buffer [78] and the weak interaction between Syt1 and SNARE proteins is unlikely to be detected in physiological conditions. Ca^{2+}-bound Syt1 has much higher binding affinity to membrane than the SNARE complex, so upon Ca^{2+} triggering, Syt1 only binds to membranes that bear anionic phospholipids [31,67]; this distinction contradicts the physiological role of Syt1–SNARE interaction.

Model 3: Syt1 causes membrane curvature

Ca^{2+}-dependent membrane insertion is the most essential and characteristic property of the C2AB domain of Syt1 [13,19]. Physiological concentration of Ca^{2+} leads to binding of the C2AB domain to negatively charged anionic phospholipids [19,31,42,79–82]. Ca^{2+}-dependent membrane binding of the C2AB domain depends on the concentration of anionic phospholipids, i.e., C2AB domain binding increases with rise in the negative charge density in membranes [83,84]. PIP_2 (−4 net charge at neutral pH) strengthens the binding affinity of Syt1 [31]. Ca^{2+}-binding to aspartate residues in the C2AB domain caused dramatic change in the surface electrostatic potential of the C2AB domain; as a result the positive electrostatic potential of the C2AB domain is attracted to anionic phospholipids, i.e., phosphatidylserine (PS) provides the complete coordination site for Ca^{2+} [25,85], and Ca^{2+} functions as an electrostatic switch that turns off repulsion between anionic phospholipids and acidic residues in the C2AB domain [64]. PIP_2 efficiently potentiates Ca^{2+}-dependent binding of C2AB domain to the target membranes [76,84,86,87] and increases the speed of response of Syt1 insertion by steering the membrane-penetration activity of Syt1 [86]. The polybasic region of the C2B domain (KKKK, 324–327; Fig. 1) increases sensitivity to Ca^{2+} by interacting with PIP_2 in the plasma membrane [26,28–31,86,88–90]. Collectively, the Ca^{2+}-binding loops of Syt1 are inserted to the plasma membrane and the polybasic region of the C2B domain interacts with $PI(4,5)P_2$ to synergistically strengthen the binding affinity of Syt1 and increase the dwell time of Syt1 in the plasma membrane [31].

Mutation in the Ca^{2+}-binding sites of Syt1 disrupts synchronous release [83,91], whereas gain-of-function mutation of Syt1 increases Ca^{2+} sensitivity of release [92]; these results agree with *in vitro* data, and support the hypothesis that Ca^{2+}-dependent membrane insertion of Syt1 is critical for Ca^{2+}-dependent vesicle fusion. Additionally, mutations in

the polybasic region of the C2B domain still show Ca^{2+}-dependent exocytosis, but reduced Ca^{2+}-sensitivity of release [88,89].

Two hydrophobic residues in the Ca^{2+}-binding loops of each C2 domain of Syt1 are partially inserted into the inner leaflet of the plasma membrane (~ 10 Å deep) [81]; this action may induce local membrane deformation in the plasma membrane [27,93]. The increased dwell time of Syt1 and deepened insertion of Syt1 into the membrane may destabilize the bilayer and thus contribute to overcome the energy barrier for fusion [31]. The C2B domain of Syt1 causes positive curvature in the plasma membrane in a Ca^{2+}-dependent manner by sequestering PS [94]. Local curvature mediated by membrane insertion of Syt1 may facilitate SNARE-mediated vesicle fusion by lowering the energy barrier of the intermediates including close membrane apposition, fusion stalk formation, and fusion pore openings (Model 3, Figs 2and 3D) [95–98]. The curvature of plasma membrane might decrease the membrane repulsion force [95,99], and the membrane repulsion becomes minimized by large reduction of the contact area of the two opposing membranes. The energy barrier to stalk formation also decreases because the positive curvature could lower the membrane bending energy required to form a stalk. In addition, the insertion of C2 domains and consequent membrane dimpling increase membrane tension that decreases the energy barrier for both the hemifusion state and fusion pore opening [100].

High concentration of the C2AB domain of Syt1 (~10 μM) in the presence of Ca^{2+} tubulates liposomes; this tubulation might result from curvature formation by the C2AB domain [27,93]. However, high concentration of the C2AB fragment is required for tubulation of liposomes. Membrane tubulation by the C2AB domain was only observed by using negative-stain electron microscopy, but not by cryo-electron microscopy [26,101]. Local positive curvature induced by C2AB insertion would facilitate fusion, but local curvature formation is mainly predicted by theoretical simulation [98]; no experiment has yet demonstrated that full-length Syt1 induces endogenous local curvature and membrane protrusion at the area of vesicle docking upon Ca^{2+} triggering. Novel techniques and approaches are expected to show the molecular mechanisms by which Syt1 causes membrane bucking.

Model 4: Syt1 causes membrane bridging

Syt1 cross-links vesicles to the plasma membrane by interactions of the C2A and C2B domain with two different membranes. Before Ca^{2+} arrival, the acidic Ca^{2+}-binding sites of these domains repel membranes, and this repulsion inhibits membrane fusion [26]. After Ca^{2+} triggering, the C2A and C2B domains bind to opposing membranes, thereby bridging the vesicle and plasma membrane [26,102]. The C2A domain is inserted into the vesicle membrane, but the polybasic region in the C2B domain interacts selectively with PIP_2, so the C2B domain binds to the plasma membrane [103] (Model 4, Fig. 2).

The linker between the C2A and C2B domain is so flexible that the two C2 domains bridge opposing membrane at ~ 4 nm distance, thereby facilitating vesicle fusion [81,103]. Membrane bridging by Syt1 may be further classified to parallel or antiparallel orientations of two C2 domains, i.e., into an oligomerization model and a direct-bridging model [101]; the linking depends on Sty1 interactions with the vesicle and the plasma membrane [32,73]. Parallel and antiparallel orientations of two C2 domains might have different functions, i.e., parallel orientations of the C2A and C2B domain to the plasma membrane trigger vesicle fusion, whereas antiparallel configuration of domains clamp vesicle fusion [104].

The SNARE complex seems to be partially assembled at a vesicle docking state, and the C2A and C2B domains of Syt1 might face the vesicle and the plasma membrane, respectively. The partial SNARE zippering brings two opposing membrane into close proximity ~ 2–4 nm before Ca^{2+} influx [105], then upon Ca^{2+} influx, Syt1 causes membrane bridging up to ~ 4 nm, but the mechanism by which this membrane bridging can lower the energy barrier to fusion is not clear. Membrane bridging by the C2A and C2B domains might decrease the energy barrier for fusion by dehydrating the interbilayer region and stabilizing the primed state (~ 2 nm) (Figs 2 and 3D). However, the physiological ionic environment that includes ATP/Mg^{2+} disrupts interaction of the C2AB domain with the vesicle membrane [76,106]; these results are evidence that the C2AB domain is only inserted into the PIP_2-containing plasma membrane to trigger vesicle fusion, not into the vesicle membrane, and may thus refute this model of membrane bridging by the C2AB domains.

Model 5: Syt1 regulates vesicle-plasma membrane distance

The energy barriers for vesicle fusion include dehydration of the phospholipids head groups and steric hindrance; for two opposing membrane to contact, proteins must be moved out of the way. Electrostatic repulsion between the vesicle and plasma membrane might contribute to this energy barrier. The vesicle

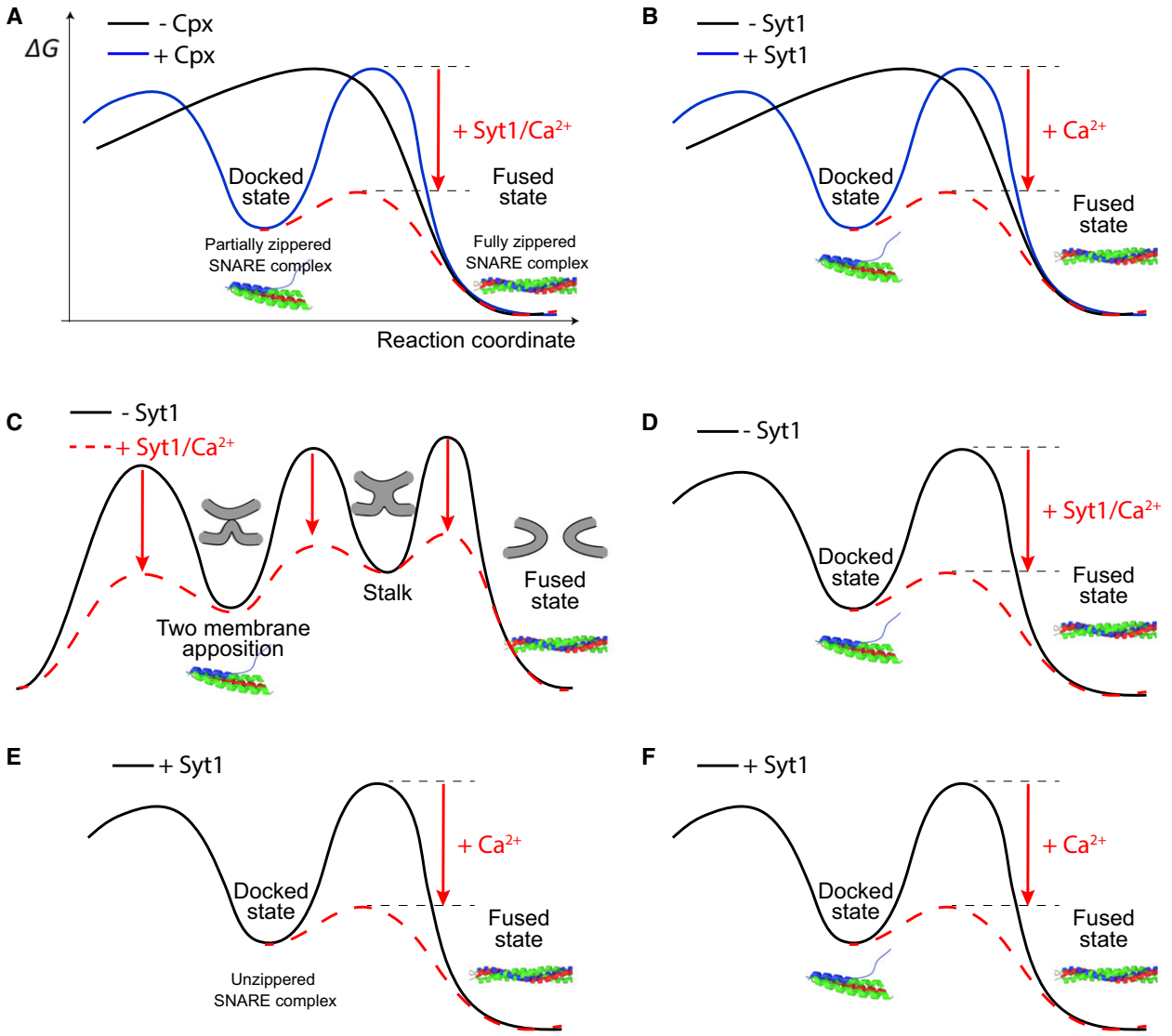

Fig. 3. Energy landscapes of six molecular models of Syt1. (A) Syt1-mediated SNARE unclamping by displacing complexin. Syt1/Ca^{2+} decreases the energy barrier by triggering full SNARE complex formation. (B) Syt1-mediated SNARE clamping. Ca^{2+} binding to Syt1 induces the full SNARE complex formation to decrease the energy barrier for membrane fusion. (C) Membrane curvature formation. Syt1/Ca^{2+} decreases the energy barriers for membrane apposition, stalk formation, and membrane fusion by positive local curvature formation. (D) Membrane bridging. The docked state results from partially zippered SNARE complex formation. Syt1/Ca^{2+} brings the two membranes into close proximity and reduces the energy barrier. (E) Distance regulator. No partial assembly of SNARE proteins at the vesicle docking state. Syt1 controls the distance between the vesicle and plasma membrane and Syt1/Ca^{2+} decreases the energy barrier by bringing two opposing membrane into close proximity. (F) Circular oligomerization of Syt1. C2B oligomerization is released by Ca^{2+} binding to the C2B domain and the dissociation of Syt1 ring decreases the energy barrier for fusion pore opening.

membrane contains the negatively charged phospholipids PS and PI (phosphatidylinositol) [107]; both have −1 net charge, whereas the plasma membrane has PS and PIP_2. Therefore, repulsion between two opposing membrane might block vesicle docking and fusion. This model suggests that SNARE proteins might not be preassembled before Ca^{2+} increase, and that Syt1 contacts the plasma membrane *via* the polybasic region [75]; this is the major difference from Model 4 (Figs 2 and 3). The polybasic region in the C2B domain is responsible for Ca^{2+}-independent binding to PIP_2, and might be involved in vesicle docking [26–32,82,86,88,90]. In a vesicle docking state, electrostatic repulsion expands the distance between two membranes, so SNARE proteins have little chance to meet. Instead, the C2AB domain of Syt1 extends to

interact with the plasma membrane *via* the polybasic region [75]. The polybasic region of the C2B domain tethers two opposing membranes, but electrostatic repulsion prevent SNARE assembly [75] (Model 5, Figs 2 and 3E). Liposomes that contain membrane-anchored Syt1 keep two membranes far (~ 7–8 nm) apart in the absence of Ca^{2+} [108], and thereby block SNARE zippering.

Upon Ca^{2+} binding, Syt1 functions as a 'charge bridge' to bring the two membranes closer together, thus initiating the SNARE nucleation and SNARE-mediated membrane fusion to occur; Syt1 functions as a distance regulator [75]. However, vesicle docking at distance of ~ 8 nm seems unlikely because synaptic vesicles are tightly docked < 2 nm from the active zone and Munc13 and other SNARE regulatory proteins might induce tight vesicle docking *in vivo* [109].

Model 6: Syt1 undergoes circular oligomerization

This model attempts to explain structural studies, and planar ring-shaped Syt1 oligomers that are observed using electron microscopy [106,110,111]. This polymerized oligomerization is conducted by oligomers of the C2B domains. The ring structure is 20–35 nm in diameter, and is composed of 12–20 Syt1 copies [110]. Syt1 rings form on the plasma membrane before Ca^{2+} triggering, and thereby block and clamp the full SNARE zippering and vesicle fusion [106]. Ca^{2+} dissociates the ring oligomerization of Syt1, thus allowing the full SNARE zippering and vesicle fusion [106,110,111] (Figs 2 and 3F).

This buttressed-ring of Syt1 might include the MUN domains of Munc13, i.e., an outer ring composed of six curved Munc13 'MUN' domains surrounds a stable inner ring of Syt1 oligomers, and thereby form a hexameric symmetrical ring structure that clamps SNARE zippering [112]. Circular oligomerization of Syt1 might be consistent with the crystal structure of two Syt1—one SNARE complex [59] in which one C2B domain coordinates the primary site (complexin-independent) and another C2B domain coordinates the tripartite site (complexin-dependent) of the SNARE complex.

Currently, no direct evidence has shown circular oligomerization of Syt1 *in vivo* at the active zone. Visualizing the buttressed rings of Syt1 in presynaptic neurons remains a topic of further study. Although Syt1 mutations that disrupt the ring structure block Syt1 function, the circular oligomerization ring structure of Syt1 is preferentially formed at low ionic strength and reduced at physiological ionic strength [110], i.e., 15 mM KCl for Syt1 ring oligomers [110] and 20 mM KCl for fluorescence correlation spectroscopy experiments [111]. Nevertheless, this model might describe the geometrical localization between SNARE/Syt1 and the fusion pore.

Discussion

The diversity of Syt1 functions *in vitro* can result from different experimental conditions. Here we describe some experiments to reduce the diversity of Syt1 functions caused by experimental artefacts. 1) *In vitro* fusion data might vary depending on whether full-length Syt1 or the C2AB domain is used for fusion assay. The C2AB domain fails to rescue the phenotype of Syt1 knockout [113] and only full-length Syt1, not the C2AB domain, efficiently reproduces Ca^{2+}-dependent fusion in the *in vitro* reconstitution system [76]. High concentration of the C2AB domain causes clustering and aggregation of liposomes and affect fusion independently of Syt1 activity. 2) Physiological Ca^{2+} concentrations should be used to test Syt1 functions. Ca^{2+} has a biphasic effect to trigger vesicle fusion *in vitro* and in chromaffin cells [67,114], i.e., 10 ~ 100 μM Ca^{2+} increases vesicle fusion, whereas 0.1 ~ 10 mM Ca^{2+} reverses it. Ca^{2+} dose–response curve is an important control experiment to ensure whether or not Ca^{2+}-dependent fusion is mediated by Syt1. 3) A normal physiological ionic environment including ATP/Mg^{2+} should be used. Syt1 interactions with membrane or SNARE proteins are tightly regulated by the electrostatic effect, and thus Syt1 function and activity vary depending on the ionic strength and salt concentration. Although the effects of low salt condition may be considered because of macromolecular crowding of these proteins [115], Syt1 activity must be confirmed *in vitro* using physiological buffer to reduce the nonspecific effect. SNARE proteins together with SNARE regulatory proteins such as Munc13, Munc18, and even NSF/alpha-SNAP create a more realistic reconstitution system to study the Syt1 function (reviewed in [116]).

In a physiological ionic environment, the main binding partner of Syt1 is most likely a membrane that contains anionic phospholipids, rather than the SNARE complex, and the polybasic region of the C2B domain may be responsible for PIP_2 interaction before Ca^{2+} triggering. The possibility still exists that Syt1 interacts with SNARE proteins when vesicles are tightly docked within 0–2 nm of the plasma membrane [109] and the SNARE complex seems to be at least partially assembled. Furthermore, membrane deformation by Ca^{2+}-bound Syt1 might contribute to overcome the energy barrier to fusion. These six models of Syt1 may be compatible and applicable at different

times and in different places, and data obtained using advanced techniques may spawn new models. It is also worth noting that some of the differences between *in vitro* biochemical assays of fusion and *in vivo* neuronal exocytosis may be due indirectly to Syt1 changes in vesicle recycling and cargo sorting, but this Review focuses explicitly on the role of Syt1 in exocytosis.

Conclusions and perspectives

Since the discovery and cloning of Syt1, its mechanisms have been examined intensively. However, molecular models of how Syt1 evokes vesicle fusion remain controversial. Syt1 interacts with SNARE proteins [39] and anionic plasma membrane [19], but the mechanisms have not yet been determined. Major debates are 1) whether Syt1 binds to the SNARE complex or to the membrane or to both [77] in a vesicle docking state in a physiological ionic environment that include ATP/Mg^{2+}, 2) how Syt1, together with complexin, controls SNARE clamping to restrict spontaneous fusion before Ca^{2+} rise, 3) whether complexin might be a SNARE clamping factor, and if not, how vesicle fusion stops before Ca^{2+} increase, and 4) how Syt1 unclamps SNARE zippering to trigger Ca^{2+}-dependent membrane fusion. We have reviewed six molecular models of Syt1 and discussed the debate about each model. Advances in technologies of cryo-electron microscopy, single-molecule measurements, *in vitro* fusion assay, nanodisc fusion assays for fusion pore nucleation, and force spectroscopy such as optical tweezers, magnetic tweezers and atomic force microscopy will help to increase understanding of the molecular mechanism by which Syt1 triggers vesicle fusion.

Acknowledgments

We apologize to many colleagues whose work could not be cited and discussed due to space limitations. This work was supported by EMBO Installation Grant (IG Project Number 3265 to YP) and by Basic Science Research Program through the National Research Foundation of Korea (NRF) from the Ministry of Education (NRF-2017R1A6A3A03010747 to JKR).

References

1 Martin TF (1994) The molecular machinery for fast and slow neurosecretion. *Curr Opin Neurobiol* **4**, 626–632.
2 Park Y and Kim KT (2009) Short-term plasticity of small synaptic vesicle (SSV) and large dense-core vesicle (LDCV) exocytosis. *Cell Signal* **21**, 1465–1470.
3 De Camilli P and Jahn R (1990) Pathways to regulated exocytosis in neurons. *Annu Rev Physiol* **52**, 625–645.
4 Pecot-Dechavassine M and Brouard MO (1997) Large dense-core vesicles at the frog neuromuscular junction: characteristics and exocytosis. *J Neurocytol* **26**, 455–465.
5 Crivellato E, Nico B and Ribatti D (2005) Ultrastructural evidence of piecemeal degranulation in large dense-core vesicles of brain neurons. *Anat Embryol (Berl)* **210**, 25–34.
6 Nitsch C and Rinne U (1981) Large dense-core vesicle exocytosis and membrane recycling in the mossy fibre synapses of the rabbit hippocampus during epileptiform seizures. *J Neurocytol* **10**, 201–209.
7 Winkler H (1993) The adrenal chromaffin granule: a model for large dense core vesicles of endocrine and nervous tissue. *J Anat* **183** (Pt 2), 237–252.
8 Martin TF (2003) Tuning exocytosis for speed: fast and slow modes. *Biochim Biophys Acta* **1641**, 157–165.
9 Littleton JT, Stern M, Schulze K, Perin M and Bellen HJ (1993) Mutational analysis of Drosophila synaptotagmin demonstrates its essential role in Ca (2 +)-activated neurotransmitter release. *Cell* **74**, 1125–1134.
10 Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF and Sudhof TC (1994) Synaptotagmin I: a major Ca2 + sensor for transmitter release at a central synapse. *Cell* **79**, 717–727.
11 Voets T, Moser T, Lund PE, Chow RH, Geppert M, Sudhof TC and Neher E (2001) Intracellular calcium dependence of large dense-core vesicle exocytosis in the absence of synaptotagmin I. *Proc Natl Acad Sci USA* **98**, 11680–11685.
12 Sorensen JB, Fernandez-Chacon R, Sudhof TC and Neher E (2003) Examining synaptotagmin 1 function in dense core vesicle exocytosis under direct control of Ca2 + . *J Gen Physiol* **122**, 265–276.
13 Perin MS, Fried VA, Mignery GA, Jahn R and Sudhof TC (1990) Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. *Nature* **345**, 260–263.
14 Katz B and Miledi R (1967) Ionic requirements of synaptic transmitter release. *Nature* **215**, 651.
15 Llinas R, Steinberg IZ and Walton K (1981) Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. *Biophys J* **33**, 323–351.
16 Sabatini BL and Regehr WG (1996) Timing of neurotransmission at fast synapses in the mammalian brain. *Nature* **384**, 170–172.
17 Walter AM, Groffen AJ, Sorensen JB and Verhage M (2011) Multiple Ca2 + sensors in secretion: teammates, competitors or autocrats? *Trends Neurosci* **34**, 487–497.
18 Kasai H, Takahashi N and Tokumaru H (2012) Distinct initial SNARE configurations underlying

the diversity of exocytosis. *Physiol Rev* **92**, 1915–1964.
19 Brose N, Petrenko AG, Sudhof TC and Jahn R (1992) Synaptotagmin: a calcium sensor on the synaptic vesicle surface. *Science* **256**, 1021–1025.
20 Craxton M (2007) Evolutionary genomics of plant genes encoding N-terminal-TM-C2 domain proteins and the similar FAM62 genes and synaptotagmin genes of metazoans. *BMC Genom* **8**, 259.
21 Xu J, Mashimo T and Sudhof TC (2007) Synaptotagmin-1, -2, and -9: Ca(2 +) sensors for fast release that specify distinct presynaptic properties in subsets of neurons. *Neuron* **54**, 567–581.
22 Schonn JS, Maximov A, Lao Y, Sudhof TC and Sorensen JB (2008) Synaptotagmin-1 and -7 are functionally overlapping Ca2 + sensors for exocytosis in adrenal chromaffin cells. *Proc Natl Acad Sci USA* **105**, 3998–4003.
23 Fernandez I, Arac D, Ubach J, Gerber SH, Shin O, Gao Y, Anderson RG, Sudhof TC and Rizo J (2001) Three-dimensional structure of the synaptotagmin 1 C2B-domain: synaptotagmin 1 as a phospholipid binding machine. *Neuron* **32**, 1057–1069.
24 Sutton RB, Davletov BA, Berghuis AM, Sudhof TC and Sprang SR (1995) Structure of the first C2 domain of synaptotagmin I: a novel Ca2 + /phospholipid-binding fold. *Cell* **80**, 929–938.
25 Ubach J, Zhang X, Shao X, Sudhof TC and Rizo J (1998) Ca2 + binding to synaptotagmin: how many Ca2 + ions bind to the tip of a C2-domain? *EMBO J* **17**, 3921–3930.
26 Arac D, Chen X, Khant HA, Ubach J, Ludtke SJ, Kikkawa M, Johnson AE, Chiu W, Sudhof TC and Rizo J (2006) Close membrane-membrane proximity induced by Ca(2 +)-dependent multivalent binding of synaptotagmin-1 to phospholipids. *Nat Struct Mol Biol* **13**, 209–217.
27 Martens S, Kozlov MM and McMahon HT (2007) How synaptotagmin promotes membrane fusion. *Science* **316**, 1205–1208.
28 van den Bogaart G, Meyenberg K, Diederichsen U and Jahn R (2012) Phosphatidylinositol 4,5-bisphosphate increases Ca2 + affinity of synaptotagmin-1 by 40-fold. *J Biol Chem* **287**, 16447–16453.
29 Wang Z, Liu H, Gu Y and Chapman ER (2011) Reconstituted synaptotagmin I mediates vesicle docking, priming, and fusion. *J Cell Biol* **195**, 1159–1170.
30 Vrljic M, Strop P, Hill RC, Hansen KC, Chu S and Brunger AT (2011) Post-translational modifications and lipid binding profile of insect cell-expressed full-length mammalian synaptotagmin 1. *Biochemistry* **50**, 9998–10012.
31 Perez-Lara A, Thapa A, Nyenhuis SB, Nyenhuis DA, Halder P, Tietzel M, Tittmann K, Cafiso DS and Jahn R (2016) PtdInsP2 and PtdSer cooperate to trap synaptotagmin-1 to the plasma membrane in the presence of calcium. *Elife* **5**, e15886.
32 Vennekate W, Schroder S, Lin CC, van den Bogaart G, Grunwald M, Jahn R and Walla PJ (2012) Cis- and trans-membrane interactions of synaptotagmin-1. *Proc Natl Acad Sci USA* **109**, 11037–11042.
33 Sollner T, Bennett MK, Whiteheart SW, Scheller RH and Rothman JE (1993) A protein assembly-disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* **75**, 409–418.
34 Schiavo G, Stenbeck G, Rothman JE and Sollner TH (1997) Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. *Proc Natl Acad Sci USA* **94**, 997–1001.
35 Rickman C and Davletov B (2003) Mechanism of calcium-independent synaptotagmin binding to target SNAREs. *J Biol Chem* **278**, 5501–5504.
36 Masumoto T, Suzuki K, Ohmori I, Michiue H, Tomizawa K, Fujimura A, Nishiki T and Matsui H (2012) Ca(2 +)-independent syntaxin binding to the C (2)B effector region of synaptotagmin. *Mol Cell Neurosci* **49**, 1–8.
37 Chicka MC, Hui E, Liu H and Chapman ER (2008) Synaptotagmin arrests the SNARE complex before triggering fast, efficient membrane fusion in response to Ca2 + . *Nat Struct Mol Biol* **15**, 827–835.
38 Gaffaney JD, Dunning FM, Wang Z, Hui E and Chapman ER (2008) Synaptotagmin C2B domain regulates Ca2 + -triggered fusion *in vitro*: critical residues revealed by scanning alanine mutagenesis. *J Biol Chem* **283**, 31763–31775.
39 Bennett MK, Calakos N and Scheller RH (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* **257**, 255–259.
40 Chapman ER, Hanson PI, An S and Jahn R (1995) Ca2 + regulates the interaction between synaptotagmin and syntaxin 1. *J Biol Chem* **270**, 23667–23671.
41 Kim JY, Choi BK, Choi MG, Kim SA, Lai Y, Shin YK and Lee NK (2012) Solution single-vesicle assay reveals PIP2-mediated sequential actions of synaptotagmin-1 on SNAREs. *EMBO J* **31**, 2144–2155.
42 Li C, Ullrich B, Zhang JZ, Anderson RG, Brose N and Sudhof TC (1995) Ca(2 +)-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature* **375**, 594–599.

43 Choi UB, Strop P, Vrljic M, Chu S, Brunger AT and Weninger KR (2010) Single-molecule FRET-derived model of the synaptotagmin 1-SNARE fusion complex. *Nat Struct Mol Biol* **17**, 318–324.

44 Dai H, Shen N, Arac D and Rizo J (2007) A quaternary SNARE-synaptotagmin-Ca2 + -phospholipid complex in neurotransmitter release. *J Mol Biol* **367**, 848–863.

45 Giraudo CG, Garcia-Diaz A, Eng WS, Chen Y, Hendrickson WA, Melia TJ and Rothman JE (2009) Alternative zippering as an on-off switch for SNARE-mediated fusion. *Science* **323**, 512–516.

46 Xue M, Reim K, Chen X, Chao HT, Deng H, Rizo J, Brose N and Rosenmund C (2007) Distinct domains of complexin I differentially regulate neurotransmitter release. *Nat Struct Mol Biol* **14**, 949–958.

47 Maximov A, Tang J, Yang X, Pang ZP and Sudhof TC (2009) Complexin controls the force transfer from SNARE complexes to membranes in fusion. *Science* **323**, 516–521.

48 Tang J, Maximov A, Shin OH, Dai H, Rizo J and Sudhof TC (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. *Cell* **126**, 1175–1187.

49 Trimbuch T and Rosenmund C (2016) Should I stop or should I go? The role of complexin in neurotransmitter release *Nat Rev Neurosci* **17**, 118–125.

50 Xue M, Craig TK, Xu J, Chao HT, Rizo J and Rosenmund C (2010) Binding of the complexin N terminus to the SNARE complex potentiates synaptic-vesicle fusogenicity. *Nat Struct Mol Biol* **17**, 568–575.

51 Snead D, Wragg RT, Dittman JS and Eliezer D (2014) Membrane curvature sensing by the C-terminal domain of complexin. *Nat Commun* **5**, 4955.

52 Zdanowicz R, Kreutzberger A, Liang B, Kiessling V, Tamm LK and Cafiso DS (2017) Complexin binding to membranes and acceptor t-SNAREs explains its clamping effect on fusion. *Biophys J* **113**, 1235–1250.

53 Trimbuch T, Xu J, Flaherty D, Tomchick DR, Rizo J and Rosenmund C (2014) Re-examining how complexin inhibits neurotransmitter release. *Elife* **3**, e02391.

54 Radoff DT, Dong Y, Snead D, Bai J, Eliezer D and Dittman JS (2014) The accessory helix of complexin functions by stabilizing central helix secondary structure. *Elife* **3**, e04553.

55 Yang X, Kaeser-Woo YJ, Pang ZP, Xu W and Sudhof TC (2010) Complexin clamps asynchronous release by blocking a secondary Ca(2 +) sensor via its accessory alpha helix. *Neuron* **68**, 907–920.

56 Rickman C, Archer DA, Meunier FA, Craxton M, Fukuda M, Burgoyne RD and Davletov B (2004) Synaptotagmin interaction with the syntaxin/SNAP-25 dimer is mediated by an evolutionarily conserved motif and is sensitive to inositol hexakisphosphate. *J Biol Chem* **279**, 12574–12579.

57 Brewer KD, Bacaj T, Cavalli A, Camilloni C, Swarbrick JD, Liu J, Zhou A, Zhou P, Barlow N, Xu J *et al.* (2015) Dynamic binding mode of a Synaptotagmin-1-SNARE complex in solution. *Nat Struct Mol Biol* **22**, 555–564.

58 Zhou Q, Lai Y, Bacaj T, Zhao M, Lyubimov AY, Uervirojnangkoorn M, Zeldin OB, Brewster AS, Sauter NK, Cohen AE *et al.* (2015) Architecture of the synaptotagmin-SNARE machinery for neuronal exocytosis. *Nature* **525**, 62–67.

59 Zhou Q, Zhou P, Wang AL, Wu D, Zhao M, Sudhof TC and Brunger AT (2017) The primed SNARE-complexin-synaptotagmin complex for neuronal exocytosis. *Nature* **548**, 420–425.

60 Bai J, Wang CT, Richards DA, Jackson MB and Chapman ER (2004) Fusion pore dynamics are regulated by synaptotagmin*t-SNARE interactions. *Neuron* **41**, 929–942.

61 Zhang X, Kim-Miller MJ, Fukuda M, Kowalchyk JA and Martin TF (2002) Ca2 + -dependent synaptotagmin binding to SNAP-25 is essential for Ca2 + -triggered exocytosis. *Neuron* **34**, 599–611.

62 Bhalla A, Chicka MC, Tucker WC and Chapman ER (2006) Ca(2 +)-synaptotagmin directly regulates t-SNARE function during reconstituted membrane fusion. *Nat Struct Mol Biol* **13**, 323–330.

63 Bhalla A, Tucker WC and Chapman ER (2005) Synaptotagmin isoforms couple distinct ranges of Ca2 + , Ba2 + , and Sr2 + concentration to SNARE-mediated membrane fusion. *Mol Biol Cell* **16**, 4755–4764.

64 Shao X, Li C, Fernandez I, Zhang X, Sudhof TC and Rizo J (1997) Synaptotagmin-syntaxin interaction: the C2 domain as a Ca2 + -dependent electrostatic switch. *Neuron* **18**, 133–142.

65 Lynch KL, Gerona RR, Larsen EC, Marcia RF, Mitchell JC and Martin TF (2007) Synaptotagmin C2A loop 2 mediates Ca2 + -dependent SNARE interactions essential for Ca2 + -triggered vesicle exocytosis. *Mol Biol Cell* **18**, 4957–4968.

66 Lynch KL, Gerona RR, Kielar DM, Martens S, McMahon HT and Martin TF (2008) Synaptotagmin-1 utilizes membrane bending and SNARE binding to drive fusion pore expansion. *Mol Biol Cell* **19**, 5093–5103.

67 Park Y, Seo JB, Fraind A, Perez-Lara A, Yavuz H, Han K, Jung SR, Kattan I, Walla PJ, Choi M *et al.* (2015) Synaptotagmin-1 binds to PIP(2)-containing membrane but not to SNAREs at physiological ionic strength. *Nat Struct Mol Biol* **22**, 815–823.

68 Littleton JT, Stern M, Perin M and Bellen HJ (1994) Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in

Drosophila synaptotagmin mutants. *Proc Natl Acad Sci USA* **91**, 10888–10892.
69 DiAntonio A and Schwarz TL (1994) The effect on synaptic physiology of synaptotagmin mutations in Drosophila. *Neuron* **12**, 909–920.
70 Pang ZP, Sun J, Rizo J, Maximov A and Sudhof TC (2006) Genetic analysis of synaptotagmin 2 in spontaneous and Ca2 + -triggered neurotransmitter release. *EMBO J* **25**, 2039–2050.
71 Xu J, Pang ZP, Shin OH and Sudhof TC (2009) Synaptotagmin-1 functions as a Ca2 + sensor for spontaneous release. *Nat Neurosci* **12**, 759–766.
72 Burgalossi A, Jung S, Meyer G, Jockusch WJ, Jahn O, Taschenberger H, O'Connor VM, Nishiki T, Takahashi M, Brose N *et al*. (2010) SNARE protein recycling by alphaSNAP and betaSNAP supports synaptic vesicle priming. *Neuron* **68**, 473–487.
73 Lai Y and Shin YK (2012) The importance of an asymmetric distribution of acidic lipids for synaptotagmin 1 function as a Ca2 + sensor. *Biochem J* **443**, 223–229.
74 Malsam J, Parisotto D, Bharat TA, Scheutzow A, Krause JM, Briggs JA and Sollner TH (2012) Complexin arrests a pool of docked vesicles for fast Ca2 + -dependent release. *EMBO J* **31**, 3270–3281.
75 van den Bogaart G, Thutupalli S, Risselada JH, Meyenberg K, Holt M, Riedel D, Diederichsen U, Herminghaus S, Grubmuller H and Jahn R (2011) Synaptotagmin-1 may be a distance regulator acting upstream of SNARE nucleation. *Nat Struct Mol Biol* **18**, 805–812.
76 Park Y, Hernandez JM, van den Bogaart G, Ahmed S, Holt M, Riedel D and Jahn R (2012) Controlling synaptotagmin activity by electrostatic screening. *Nat Struct Mol Biol* **19**, 991–997.
77 Wang S, Li Y and Ma C (2016) Synaptotagmin-1 C2B domain interacts simultaneously with SNAREs and membranes to promote membrane fusion. *Elife* **5**, e14211.
78 Honig BH, Hubbell WL and Flewelling RF (1986) Electrostatic interactions in membranes and proteins. *Annu Rev Biophys Biophys Chem* **15**, 163–193.
79 Davletov BA and Sudhof TC (1993) A single C2 domain from synaptotagmin I is sufficient for high affinity Ca2 + /phospholipid binding. *J Biol Chem* **268**, 26386–26390.
80 Chapman ER and Davis AF (1998) Direct interaction of a Ca2 + -binding loop of synaptotagmin with lipid bilayers. *J Biol Chem* **273**, 13995–14001.
81 Herrick DZ, Sterbling S, Rasch KA, Hinderliter A and Cafiso DS (2006) Position of synaptotagmin I at the membrane interface: cooperative interactions of tandem C2 domains. *Biochemistry* **45**, 9668–9674.
82 Schiavo G, Gu QM, Prestwich GD, Sollner TH and Rothman JE (1996) Calcium-dependent switching of the specificity of phosphoinositide binding to synaptotagmin. *Proc Natl Acad Sci USA* **93**, 13327–13332.
83 Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C and Sudhof TC (2001) Synaptotagmin I functions as a calcium regulator of release probability. *Nature* **410**, 41–49.
84 Zhang X, Rizo J and Sudhof TC (1998) Mechanism of phospholipid binding by the C2A-domain of synaptotagmin I. *Biochemistry* **37**, 12395–12403.
85 Murray D and Honig B (2002) Electrostatic control of the membrane targeting of C2 domains. *Mol Cell* **9**, 145–154.
86 Bai J, Tucker WC and Chapman ER (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.
87 Radhakrishnan A, Stein A, Jahn R and Fasshauer D (2009) The Ca2 + affinity of synaptotagmin 1 is markedly increased by a specific interaction of its C2B domain with phosphatidylinositol 4,5-bisphosphate. *J Biol Chem* **284**, 25749–25760.
88 Li L, Shin OH, Rhee JS, Arac D, Rah JC, Rizo J, Sudhof T and Rosenmund C (2006) Phosphatidylinositol phosphates as co-activators of Ca2 + binding to C2 domains of synaptotagmin 1. *J Biol Chem* **281**, 15845–15852.
89 Loewen CA, Lee SM, Shin YK and Reist NE (2006) C2B polylysine motif of synaptotagmin facilitates a Ca2 + -independent stage of synaptic vesicle priming *in vivo*. *Mol Biol Cell* **17**, 5211–5226.
90 Kuo W, Herrick DZ, Ellena JF and Cafiso DS (2009) The calcium-dependent and calcium-independent membrane binding of synaptotagmin 1: two modes of C2B binding. *J Mol Biol* **387**, 284–294.
91 Mackler JM, Drummond JA, Loewen CA, Robinson IM and Reist NE (2002) The C(2)B Ca(2 +)-binding motif of synaptotagmin is required for synaptic transmission *in vivo*. *Nature* **418**, 340–344.
92 Rhee JS, Li LY, Shin OH, Rah JC, Rizo J, Sudhof TC and Rosenmund C (2005) Augmenting neurotransmitter release by enhancing the apparent Ca2 + affinity of synaptotagmin 1. *Proc Natl Acad Sci USA* **102**, 18664–18669.
93 Hui E, Johnson CP, Yao J, Dunning FM and Chapman ER (2009) Synaptotagmin-mediated bending of the target membrane is a critical step in Ca(2 +)-regulated fusion. *Cell* **138**, 709–721.
94 Lai AL, Tamm LK, Ellena JF and Cafiso DS (2011) Synaptotagmin 1 modulates lipid acyl chain order in lipid bilayers by demixing phosphatidylserine. *J Biol Chem* **286**, 25291–25300.

95 Chernomordik LV and Kozlov MM (2003) Protein-lipid interplay in fusion and fission of biological membranes. *Annu Rev Biochem* **72**, 175–207.
96 Marrink SJ and Mark AE (2003) The mechanism of vesicle fusion as revealed by molecular dynamics simulations. *J Am Chem Soc* **125**, 11144–11145.
97 Monck JR and Fernandez JM (1992) The exocytotic fusion pore. *J Cell Biol* **119**, 1395–1404.
98 Kozlov MM and Chernomordik LV (2002) The protein coat in membrane fusion: lessons from fission. *Traffic* **3**, 256–267.
99 Marrink SJ and Mark AE (2003) Molecular dynamics simulation of the formation, structure, and dynamics of small phospholipid vesicles. *J Am Chem Soc* **125**, 15233–15242.
100 Chanturiya A, Scaria P, Kuksenok O and Woodle MC (2002) Probing the mechanism of fusion in a two-dimensional computer simulation. *Biophys J* **82**, 3072–3080.
101 Seven AB, Brewer KD, Shi L, Jiang QX and Rizo J (2013) Prevalent mechanism of membrane bridging by synaptotagmin-1. *Proc Natl Acad Sci USA* **110**, E3243–E3252.
102 Connell E, Giniatullina A, Lai-Kee-Him J, Tavare R, Ferrari E, Roseman A, Cojoc D, Brisson AR and Davletov B (2008) Cross-linking of phospholipid membranes is a conserved property of calcium-sensitive synaptotagmins. *J Mol Biol* **380**, 42–50.
103 Herrick DZ, Kuo W, Huang H, Schwieters CD, Ellena JF and Cafiso DS (2009) Solution and membrane-bound conformations of the tandem C2A and C2B domains of synaptotagmin 1: evidence for bilayer bridging. *J Mol Biol* **390**, 913–923.
104 Bai H, Xue R, Bao H, Zhang L, Yethiraj A, Cui Q and Chapman ER (2016) Different states of synaptotagmin regulate evoked versus spontaneous release. *Nat Commun* **7**, 10971.
105 Li F, Pincet F, Perez E, Eng WS, Melia TJ, Rothman JE and Tareste D (2007) Energetics and dynamics of SNAREpin folding across lipid bilayers. *Nat Struct Mol Biol* **14**, 890–896.
106 Zanetti MN, Bello OD, Wang J, Coleman J, Cai Y, Sindelar CV, Rothman JE and Krishnakumar SS (2016) Ring-like oligomers of Synaptotagmins and related C2 domain proteins. *Elife* **5**, e17262.
107 Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P *et al*. (2006) Molecular anatomy of a trafficking organelle. *Cell* **127**, 831–846.
108 Lin CC, Seikowski J, Perez-Lara A, Jahn R, Hobartner C and Walla PJ (2014) Control of membrane gaps by synaptotagmin-Ca2 + measured with a novel membrane distance ruler. *Nat Commun* **5**, 5859.
109 Imig C, Min SW, Krinner S, Arancillo M, Rosenmund C, Sudhof TC, Rhee J, Brose N and Cooper BH (2014) The morphological and molecular nature of synaptic vesicle priming at presynaptic active zones. *Neuron* **84**, 416–431.
110 Wang J, Bello O, Auclair SM, Wang J, Coleman J, Pincet F, Krishnakumar SS, Sindelar CV and Rothman JE (2014) Calcium sensitive ring-like oligomers formed by synaptotagmin. *Proc Natl Acad Sci USA* **111**, 13966–13971.
111 Wang J, Li F, Bello OD, Sindelar CV, Pincet F, Krishnakumar SS and Rothman JE (2017) Circular oligomerization is an intrinsic property of synaptotagmin. *Elife* **6**, e27441.
112 Rothman JE, Krishnakumar SS, Grushin K and Pincet F (2017) Hypothesis - buttressed rings assemble, clamp, and release SNAREpins for synaptic transmission. *FEBS Lett* **591**, 3459–3480.
113 Lee J and Littleton JT (2015) Transmembrane tethering of synaptotagmin to synaptic vesicles controls multiple modes of neurotransmitter release. *Proc Natl Acad Sci USA* **112**, 3793–3798.
114 Knight DE and Baker PF (1982) Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. *J Membr Biol* **68**, 107–140.
115 Ganji M, Docter M, Le Grice SF and Abbondanzieri EA (2016) DNA binding proteins explore multiple local configurations during docking via rapid rebinding. *Nucleic Acids Res* **44**, 8376–8384.
116 Brunger AT, Choi UB, Lai Y, Leitz J and Zhou Q (2018) Molecular mechanisms of fast neurotransmitter release. *Annu Rev Biophys* **47**, 469–497.