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A conformational switch in complexin is required for synaptotagmin to trigger synaptic fusion

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The crystal structure of complexin bound to a prefusion SNAREpin mimetic shows that the accessory helix extends away from the SNAREpin in an 'open' conformation, binding another SNAREpin and inhibiting its assembly, to clamp fusion. In contrast, the accessory helix in the postfusion complex parallels the SNARE complex in a 'closed' conformation. Here we use targeted mutations, FRET spectroscopy and a functional assay that reconstitutes Ca^{2+}-triggered exocytosis to show that the conformational switch from open to closed in complexin is needed for synaptotagmin-Ca^{2+} to trigger fusion. Triggering fusion requires the zippering of three crucial aspartate residues in the switch region (residues 64–68) of v-SNARE. Conformational switching in complexin is integral to clamp release and is probably triggered when its accessory helix is released from its *trans*-binding to the neighboring SNAREpin, allowing the v-SNARE to complete zippering and open a fusion pore.

SNARE proteins are the core machinery driving membrane fusion between cargo-carrying vesicles and their target membranes[1–4], as v-SNAREs (anchored in the vesicle membrane) zipper into a coiled-coil four-helix bundle[5] with cognate t-SNAREs (anchored in the target membrane). In neuronal synapses, the principal SNAREs responsible for neurotransmitter release are the v-SNARE, VAMP2, which is localized on the synaptic vesicle, and the t-SNARE, a binary complex of SNAP25 with syntaxin1, localized on the presynaptic plasma membrane. VAMP2 and syntaxin1 each contribute one helix to the coiled coil, and SNAP25 contributes the other two. Fusion by the isolated synaptic SNAREs is rapid and spontaneous[6], implying that in the synapse, additional protein machinery is needed to arrest exocytosis until the secretion signal is provided by the entry of calcium ions.

One such protein is the calcium- and SNARE-binding protein synaptotagmin[7,8], which is the immediate sensor for synchronous vesicle fusion[9–11]. The SNARE complex binding protein complexin[12,13] (CPX) is equally required for synaptic transmission, functioning both positively as an activator of fusion and negatively as a clamp, to prevent fusion before the calcium signal[14–19]. We established on energetic grounds how CPX can be both a clamp and an activator of SNAREpins, as detailed in an accompanying manuscript[20].

In a preceding study, we determined the precise nature of the clamped state using X-ray crystallography together with confirmatory solution and functional studies[21]. To capture this intermediate, we used a SNARE complex containing a C-terminally truncated VAMP (termed VAMP2-60) to mimic the half-zippered v-SNARE that is trapped in the clamped state[14,18,20,22–24]. In the structure, human CPX1 binds one partially zippered SNARE complex through its central helix (CPX_{cen}, residues 48–75), while its accessory helix (CPX_{acc}, residues 26–48) extends away from this SNARE complex at ~45° to bridge to a second SNAREpin, binding its C-terminal three-helix bundle to sterically block that SNAREpin's own v-SNARE from completing its zippering. As a result of repeating these *trans*-interactions, the clamped SNAREpins are cross-linked into a rigid zigzag array[21], which is itself topologically incompatible with the opening of a fusion pore.

In this paper, we address the following question: how is fusion switched 'on' from the clamped state? A newly discovered feature of the prefusion mimetic structure is that in the clamped state, CPX_{acc} angles away from the SNAREpin to which its central helix is attached, a conformation that we refer to as open (**Fig. 1a**, cyan). This differs substantially from that observed in the fully zippered postfusion structure, where the CPX_{acc} nearly parallels the SNARE complex[25,26], which we refer to as closed (**Fig. 1a**, light cyan). Taken together, the two structures suggest that CPX_{acc} undergoes a marked reorientation as part of the mechanism (requiring calcium binding to synaptotagmin) that switches fusion on from the clamped state. In this paper, we show that the movement from the open to closed state is a molecular switch required to activate fusion from the clamped state. We report that three closely clustered aspartate residues (positions 64, 65 and 68) in the C-terminal half of VAMP2 (termed the 'aspartate switch region') must zipper into the t-SNARE for CPX to move its accessory helix from the open (clamped) to the closed (postfusion) conformation. Mutating these residues inhibits activation of fusion from the clamped state by destabilizing the open conformation.

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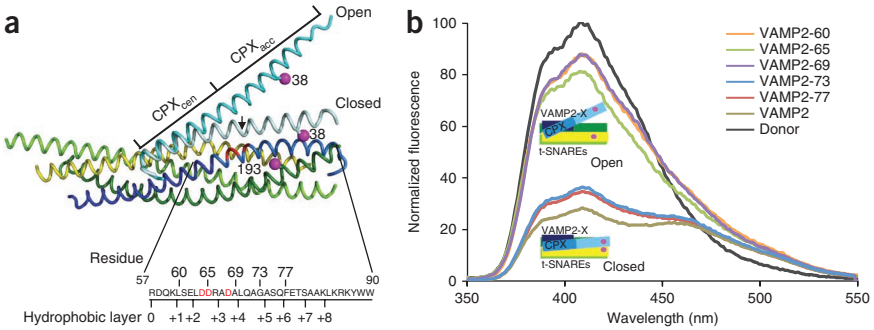

Figure 1 Zippering of one turn of VAMP2 helix triggers the switch in CPX position. (**a**) Superposition of the structures of the pre- and postfusion CPX–SNARE complexes[21,25], showing the FRET label positions. Syntaxin1 (residues 190–250) is in yellow, SNAP25 N-terminal SNARE motif (residues 10–74) is in pale green, SNAP25 C-terminal SNARE motif (residues 141–203) is in green and VAMP2 is in blue (residues 25–60 dark blue; residues 61–96 in light blue). The switch residues (Asp64, Asp65 and Asp68) are marked in red. The complexin (residues 26–73) in the prefusion complex is in cyan, and in the postfusion complex is in light cyan. The FRET label positions, residue 193 on SNAP25 and residue 38 on complexin, are marked as magenta spheres. The sequence of the C-terminal hydrophobic layer of VAMP2 (residues 57–90) with the C-terminal truncations tested in this paper (denoted by the residue number) is also shown. The black arrow in the postfusion structure references CPX residue 48, the demarcation line between CPX_{cen} and CPX_{acc}. (**b**) FRET experiments with C-terminal truncations of VAMP2. Fluorescence emission spectra of stilbene- and bimane-labeled CPX–SNARE complexes containing VAMP2-60 (residues 25–60, orange), VAMP2-65 (residues 25–65, green), VAMP2-69 (residues 25–69, purple), VAMP2-73 (residues 25–73, blue), VAMP2-77 (residues 25–77, red) and VAMP2 (residues 1–96, olive). A representative emission spectrum of a stilbene (donor)-only CPX–SNARE complex is shown in black. The donor-only spectrum was identical in all CPX–SNARE complexes.

RESULTS

Zippering one turn of VAMP2 helix triggers the CPX switch

We use here fluorescence resonance energy transfer (FRET) experiments with a stilbene-bimane donor-acceptor pair to monitor the conformational state of CPX_{acc} in CPX–SNARE complexes[21]. In this assay, residue 193 of SNAP25 is labeled with the donor dye (stilbene), and the acceptor dye (bimane) is attached at residue 38 of CPX_{acc} (**Fig. 1a**). This pair was chosen because of its sensitivity to changes in separations in the range of interest. When CPX_{acc} adopts the open conformation, the fluorescent probes are far apart, resulting in low FRET, but when CPX_{acc} moves to the closed conformation, the FRET probes are close together, resulting in a larger FRET signal. Distances obtained in solution from FRET analysis of VAMP2 constructs used in crystallization (VAMP2-60 or VAMP2) were in excellent agreement with the distances observed in the respective crystals (**Table 1**).

This FRET assay allows us to distinguish the open conformation (complexes with VAMP2 truncated at residue 60) from the closed conformation (complexes in which the cytoplasmic domain of VAMP is nearly complete, truncated at residue 96) (**Fig. 1b**). To explore what happens in complexes with VAMP2 truncations in between these two extremes, we assembled full-length CPX–SNARE complexes with the VAMP2 C terminus progressively extended beyond residue 60 (VAMP2-65, VAMP2-69, VAMP2-73 and VAMP2-77) to mimic the progressive zippering of the VAMP2 C terminus.

The FRET spectra show that CPX_{acc} adopted the open conformation in truncated CPX–SNARE complexes when the VAMP2 C terminus was extended to the +2 (VAMP2-65) or +3 (VAMP2-69) hydrophobic layers (**Fig. 1b**). In these complexes, we observe low FRET efficiency, and the distance between the probes as measured by quenching of the donor fluorescence is consistent with the distances observed in the crystal structure of the CPX–SNARE-60 complex (**Fig. 1b** and **Table 1**).

In contrast, when we extended the VAMP2 C terminus only about one turn of the helix further, to the +4 hydrophobic layer (VAMP2-73) or beyond (VAMP2-77), we observed high FRET, quantitatively corresponding to the closed conformation, in which CPX_{acc} runs parallel to the SNARE complex (**Fig. 1b**). In these complexes, the distance between donor and acceptor probes calculated from the FRET spectra is consistent with distances observed in the postfusion CPX–SNARE structure (**Table 1**).

Notably, there is an all-or-none, discrete switch from open to closed conformation when one more turn of the helix is added between VAMP2 residues 69 and 73, without hybrid spectra of the two states. This shows that zippering of VAMP2 to at least the +4 hydrophobic layer is required for the switch in CPX conformation, indicating that a discrete switch region is located in this stretch of VAMP2. The full-length 'superclamp' CPX (scCPX) was used in these and later studies in this paper for consistency and direct comparability with the crystal structure, which also used mutations in the CPX_{acc} (D27L E34F R37A) that increase binding to the t-SNARE.

Aspartate residues in the critical region of VAMP2 throw the switch

In the postfusion structure[25], CPX binds the SNARE complex in the groove between VAMP2 and syntaxin1, and the CPX_{cen} makes three distinct contacts with VAMP2. Two of these interactions, a hydrophobic contact with VAMP2 Val50 and Leu54 and a salt bridge with VAMP2 Asp57, are found in both the prefusion, clamped CPX–SNARE complex[21] and the postfusion, fully zippered CPX–SNARE complex[25] structures. A third, distinct contact region involving VAMP2 residues Asp64,

Table 1 FRET distances in CPX–SNARE complexes

Constructs	Distance between SNAP25 Asp193 and CPX Gln38 (Å)
VAMP2 deletions	
VAMP2-60	34 ± 1
VAMP2-65	33 ± 2
VAMP2-69	34 ± 1
VAMP2-73	21 ± 2
VAMP2-77	24 ± 1
VAMP2 and VAMP2 mutants (residues 1–96)	
VAMP2	20 ± 1
VAMP-4X	33 ± 1
VAMP-3xDA	32 ± 3
Measured in crystal structure	
Prefusion (VAMP2-60)	28
Postfusion (VAMP2)	18

FRET distances were determined from quenching of donor fluorescence between SNAP25 Asp193 and CPX Gln38 in CPX–SNARE complexes with VAMP2 deletions and mutants. The distances measured in the prefusion and postfusion crystal structures[21,25] are given for comparison. s.d. are reported from $n = 4$–6 independent experiments, and they reflect the reproducibility of the spectra rather than accuracy of the distance measurements.

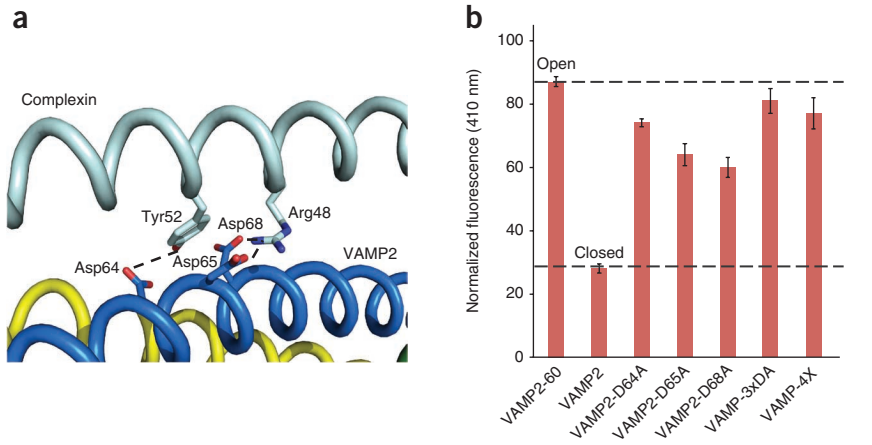

Figure 2 Aspartate residues 64, 65 and 68 on VAMP2 mediate the switch in CPX position. (**a**) Hydrogen bonding and salt bridge interactions between the switch aspartate residues 64, 65 and 68 with CPX_{cen} helix in the postfusion complex[25]. (**b**) Complexin adopts an open conformation in CPX–SNARE complexes containing either VAMP-3xDA or VAMP-4X. Donor fluorescence at 410 nm (normalized to a donor-only sample) for stilbene-bimane–labeled CPX–SNARE complexes containing VAMP2-D64A, VAMP2-D65A, VAMP2-D68A, VAMP-3xDA or VAMP-4X is shown. (The raw fluorescence emission curves are shown in **Supplementary Fig. 3**.) The donor fluorescence (at 410 nm) for VAMP2-60 (open) and VAMP2 (closed) are shown for comparison. Averages and s.d. for three or four independent experiments are shown.

Asp65 and Asp68 is present in the postfusion complex only[25] (**Fig. 2a**) because the VAMP C terminus was truncated at residue 60 in the prefusion complex[21]. These three aspartate residues are located within the +2 to +4 hydrophobic layers of VAMP2, which we identified (**Fig. 1b**) as the minimal switch region required to switch CPX_{acc} from the open to the closed conformation. They form hydrogen bonds and salt bridges to CPX_{cen} that serve to anchor the accessory helix parallel to the four-helix SNARE bundle in the closed, postfusion conformation (**Fig. 2a**).

To test the hypothesis that these contacts are needed to stabilize the closed conformation, we mutated all three switch aspartate residues to alanines in an otherwise complete VAMP2 cytoplasmic domain (residues 1–96) containing all of the VAMP residues that assemble into the four-helix SNARE bundle[5], a construct referred to as VAMP-3xDA (VAMP2 with D64A D65A D68A mutations). As expected, VAMP-3xDA fully zippers into t-SNARE, as shown by resistance to cleavage by tetanus and botulinum-B neurotoxins (**Supplementary Fig. 1a**) and the fact that VAMP-3xDA (when produced in a full-length form, containing its membrane anchor) retains its capacity to mediate fusion with its cognate synaptic t-SNARE in the liposome fusion assay (**Supplementary Fig. 1b**).

As predicted by our hypothesis, CPX_{acc} adopts the open conformation in the fully zippered CPX–SNARE-3xDA complex, and its FRET spectrum corresponds closely to those of the prefusion mimetics of CPX–SNARE in which the switch region of VAMP is deleted (**Fig. 2b** and **Table 1**, raw data in **Supplementary Fig. 2a**). The consequences of mutating individual switch aspartate residues reveal that all three are required for the full switching of the CPX_{acc}, as mutation of any of the three aspartate residues (VAMP2-D64A, VAMP2-D65A or VAMP2-D68A) destabilizes the closed conformation (**Fig. 2b**; raw data in **Supplementary Fig. 2b**). Mutating residue 64 (VAMP2-D64A) has the maximum destabilizing effect, so it might act as the internal trigger for the switch (**Fig. 2b**).

Zippering of aspartate switch residues is required for the switch

So far, we know that CPX_{acc} is trapped in the open conformation when the switch aspartates are absent from VAMP (owing to either point mutations or deletion). What about when the switch aspartates are present but not able to zipper? This would correspond more closely to what occurs physiologically before these residues have zippered. To mimic this state, we used a VAMP construct in which the normal switch aspartates are present but that carries point mutations that prevent full zippering in the C-terminal (membrane-proximal) region of VAMP.

Specifically, we used the entire VAMP2 cytoplasmic domain (residues 1–96) and introduced mutations in its C-terminal half (L70D A74R A81D L84D; collectively termed VAMP-4X) that prevent assembly of this region with syntaxin1 and SNAP25 and eliminate fusion activity (**Supplementary Fig. 1b**). However, the N-terminal half of VAMP-4X still zippers because VAMP-4X forms stable complexes with the t-SNAREs and CPX (**Supplementary Fig. 3**). The FRET spectrum of the CPX–SNARE-4X complex (**Fig. 2b** and **Table 1**; raw data in **Supplementary Fig. 2a**) is nearly identical to that of the open conformation, as present in the CPX–SNARE complex with VAMP-60 or when the switch aspartates are mutated or deleted by truncations (**Fig. 2b** and **Table 1**).

Notably, this experiment establishes that the mere presence of the VAMP2 residues Asp64, Asp65 and Asp68 is not sufficient for switching: they must also be zippered with the t-SNARE in the helical bundle in order to throw the switch from open to closed. This strongly suggests that zippering of the switch region of the v-SNARE (that is, progression of fusion beyond the clamped state) and movement of CPX_{acc} from its open to its closed arrangement are thermodynamically coupled; in other words, one cannot occur without the other.

This could also explain why CPX_{acc} adopts an open conformation in the CPX–SNARE-69 complex (**Fig. 1b** and **Table 1**). Even though the key residues required for the switch (Asp64, Asp65 and Asp68) are present in this complex, they are at the end of the truncated VAMP2, and they may not be properly zippered into the t-SNARE. Extending the VAMP2 C terminus one rung on the helix to the next hydrophobic layer (VAMP2-73) could then allow the switch region to stably zipper into the t-SNARE and switch CPX_{acc} to the closed conformation (**Fig. 1b** and **Table 1**).

Thermodynamics of the CPX conformational switch

We used isothermal titration calorimetry (ITC) to determine the energetics of the contributions of the switch aspartate residues to the open-to-closed conformational switch. We compared the thermodynamics of the binding of CPX to SNARE complexes assembled with either VAMP2 or VAMP-3xDA (**Fig. 3**). Complexin binds the VAMP2 SNARE complex with 1:1 stoichiometry and high affinity (K_d = 83 nM). Mutating the switch aspartate residues (VAMP-3xDA) did not alter the binding stoichiometry but resulted in 88% reduction in the affinity (K_d = 670 nM) (**Fig. 3** and **Table 2**), corresponding to a free energy difference of −1.3 kcal mol^{-1}. The enthalpy of the interaction of CPX with the SNARE complex was greatly reduced, from −15 kcal mol^{-1} for the VAMP-3xDA SNARE complex to −37.5 kcal mol^{-1} for the wild-type VAMP2 sequence. So the difference in the enthalpy ($\Delta\Delta H$ = −22.5 kcal mol^{-1}) is mainly from the interaction of CPX_{cen} with the switch aspartate residues on VAMP2.

In a control experiment, when we blocked the CPX_{cen} binding site on the VAMP-3xDA SNARE complex by prebinding CPX_{cen}

Table 2 Thermodynamic parameters of CPX binding to SNARE complexes

Titrant	In sample cell	Stoichiometric coefficient (N)	K_d (nM)	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔG (kcal mol^{-1})
scCPX	SNARE complex with VAMP2-WT	0.95 ± 0.01	83 ± 17	−37.5 ± 0.7	−88.6 ± 2.7	−10.1 ± 0.2
scCPX	SNARE complex with VAMP-3xDA	0.99 ± 0.04	670 ± 90	−15.0 ± 1.0	−20.1 ± 3.6	−8.8 ± 0.2
CPX$^{48–134}$	SNARE complex with VAMP-3xDA	0.98 ± 0.01	620 ± 110	−14.0 ± 1.1	−16.6 ± 3.8	−8.8 ± 0.1

Thermodynamic parameters of CPX binding to SNAREs measured by ITC. Superclamp CPX (scCPX, residues 1–134 carrying superclamp mutation D27L E34F R37A) or CPX$^{48–134}$ were titrated into assembled SNARE complexes containing VAMP2 (residues 1–96) or VAMP-3xDA (residues 1–96 with mutations D64A, D65A and D68A). The thermodynamic parameters of dissociation constant (K_d), enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) were calculated by nonlinear least-squares fit with a 'one-set-of-sites' model from the binding isotherms shown in **Figure 3**. Average and s.d. of a minimum of three independent experiments are shown.

(residues 48–134), we saw no further interaction with CPX (**Fig. 3**). We conclude that the interaction of CPX with the SNARE–3xDA complex is mediated solely by CPX$_{cen}$. This observation confirms that the SNARE–3xDA complex is fully zippered because the additional binding site for CPX$_{acc}$, that is, the C-terminal t-SNARE groove, is not available.

The switch in CPX is required for synaptotagmin to trigger fusion

To the extent that the open-to-closed conformational switch in CPX is needed to activate fusion from the clamped state, locking CPX$_{acc}$ in the open state will prevent activation of fusion and result in a persistent clamped state, which should inhibit activation of fusion by synaptotagmin and calcium ions.

To test this, we used the 'flipped' SNARE system in which cells expressing either VAMP2 or syntaxin1-SNAP25 proteins on their surface are mixed and the rate of cell-to-cell fusion is scored using light microscopy[2]. In this system, fusion occurs spontaneously unless CPX is added either as an exogenous pure protein or by endogenous gene expression and secretion. In the presence of CPX, fusion is blocked when the SNAREs are approximately half-zippered, as judged by the pattern of botulinum and tetanus neurotoxin resistance[14]. When synaptotagmin is either added back to the medium (cytoplasmic domain only) or endogenously expressed as a flipped protein, fusion is then reactivated upon addition of Ca^{2+} ions[14]. The physiological relevance of this minimal system has been established by several criteria[14]. For example, mutations in synaptotagmin that alter calcium sensitivity in mice correspondingly alter sensitivity in this reconstituted system, toxin sensitivity in the clamped state reproduces the pattern found at the neuromuscular junction and—most recently—superclamp mutations of CPX$_{acc}$ that increase clamping potency in neurons[27] also do so in this *in vitro* system[15].

We tested the activation of fusion mediated by VAMP-3xDA (VAMP2 with D64A, D65A and D68A mutations) from the clamped state by synaptotagmin and calcium and confirmed the prediction that clamp release would be impaired when the v-SNARE lacked the switch aspartate residues (**Fig. 4a**, VAMP-3xDA, green bar). The limited extent of activation that was observed with VAMP-3xDA and wild-type CPX was similar to the essentially permanent clamped state that results when wild-type SNAREs are frozen with superclamp CPX (**Fig. 4a**, scCPX, green bar). In the latter case, the open state is stabilized by stronger binding of CPX$_{acc}$ to the *trans*-t-SNARE, whereas in the former case it is the closed state that is destabilized by weaker binding of CPX$_{cen}$ to the SNARE bundle due to switch aspartate mutations. As expected, mutating individual aspartate residues in the switch region partially compromised activation in a manner reflecting their relative contributions to stabilizing the closed conformation measured by FRET (**Fig. 2b**).

As a control, we tested VAMP-3xDA in the absence of any CPX to confirm that it is intrinsically fusion competent (**Fig. 4a**, blue bars). Furthermore, we found that the clamping of fusion by wild-type CPX was identical for cells expressing VAMP2 or VAMP-3xDA (**Fig. 4a**, red bars). Because the mutations of the switch aspartates lock CPX$_{acc}$ in the open state, the fact that we see functional clamping in VAMP-3xDA is consistent with the prediction that the accessory helix exerts clamping in the open conformation[21].

To further characterize the VAMP-3xDA mutation, we analyzed the kinetics of the activation of fusion by synaptotagmin and calcium from the clamped state. We found that the overall kinetics of clamp release was similar in cells expressing VAMP-3xDA or VAMP2 (**Fig. 4b**), but the extent of activation of fusion by Ca^{2+}-synaptotagmin was limited at all time points (**Fig. 4b**). These activation curves have the same calcium requirement (EC_{50} ~100 μM, **Supplementary Fig. 4**), indicating that the effect is mainly due to an intrinsic property of

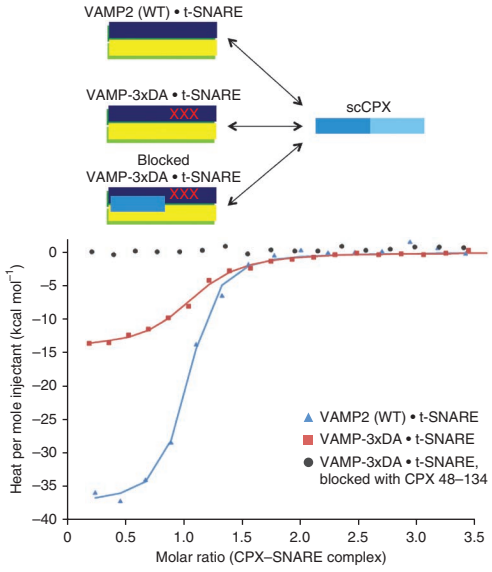

Figure 3 Interaction of CPX$_{cen}$ with aspartate residues 64, 65 and 68 on VAMP2 provides thermodynamic driving force for the switch. Calorimetric titrations of superclamp CPX (scCPX; residues 1–134 with D27L E34F R37A mutations) into assembled SNARE complexes containing t-SNAREs and either wild-type (WT) VAMP2 (blue triangles), VAMP2-3xDA (red squares) or VAMP-3xDA with the CPX$_{cen}$ binding site blocked by CPX 48–134 (black circles). The solid lines represent the best fit to the corresponding data points using a nonlinear least-squares fit with a one-set-of-sites model. The results of the fits are given in **Table 2**. All experiments were conducted in triplicate at 37 °C, and a representative thermogram is shown.

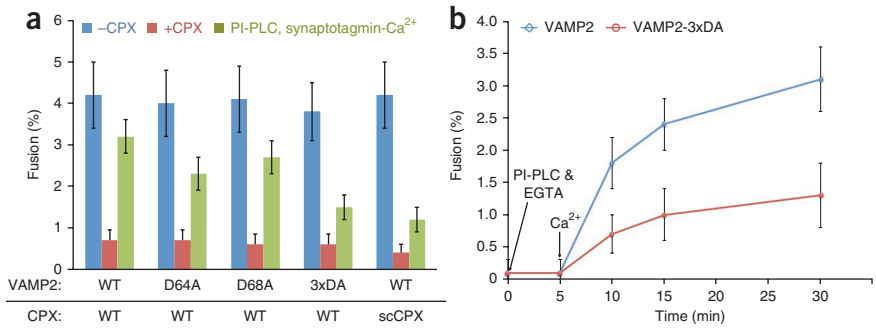

Figure 4 The switch in CPX_{acc} position is necessary for synaptotagmin-Ca^{2+} to trigger fusion. (**a**) Clamping of SNARE-mediated fusion by CPX and the reversal of the clamp by synaptotagmin-Ca^{2+} in the presence of phosphatidylinositol-specific phospholipase-C (PI-PLC) in wild-type (WT) VAMP2, VAMP2-D64A, VAMP2-D68A and VAMP-3xDA as measured in a cell-cell fusion assay. The effect of the superclamp CPX (scCPX; CPX D27L, E34F, R37A) on wild-type VAMP2 is shown for comparison. (**b**) Kinetics of the reversal of the CPX clamp by synaptotagmin-Ca^{2+}. The cell fusion recovery was carried out at 1 mM free Ca^{2+}, and the samples were fixed at the indicated time point after the addition of Ca^{2+}. Averages and s.d. of two or three independent experiments are shown.

the VAMP-3xDA mutation rather an impairment in synaptotagmin. These results clearly demonstrate that the conformational switch in CPX position is essential for synaptotagmin to trigger fusion upon arrival of the Ca^{2+} signal.

DISCUSSION

The data presented establish that a region near the middle of the VAMP2 SNARE motif must be folded with the t-SNARE in order for CPX to assume the closed conformation. This positions the key aspartate residues (Asp64, Asp65 and Asp68) to interact with the CPX central helix (**Fig. 2a**), driving CPX from its open into its closed conformation. The data also reveal that accessing the closed conformation is not required for clamping, but it is required for calcium-bound synaptotagmin to release the clamp.

Clamp release entails removing CPX_{acc} from its *trans*-t-SNARE binding site so that the *trans*-SNAREpin can complete zippering and thereby fuse the bilayers. In light of the network of interactions constituting the zigzag array[21] (**Fig. 5**), it is hard to imagine how this could occur as an isolated event. For example, if any one CPX in the array (such as the CPX emanating from SNAREpin 3 in **Fig. 5**) were to flip from open to closed, in so doing, it would necessarily pull its CPX_{acc} out of the binding pocket of its *trans*-SNAREpin across the midline of the array (number 2), where it had—up until then—been bound (**Fig. 5**). However, in actuality, this could not happen unless the switch region of the v-SNARE within SNAREpin 3 had somehow zippered up to create the binding site needed to anchor its emanating CPX_{acc}. This, in turn, is prohibited by yet another CPX_{acc} (emanating from SNAREpin 4) that plugs the path of the v-SNARE within SNAREpin 3 (**Fig. 5**). This suggests that the zippering of the VAMP2 C terminus and the conformational switch in CPX must both occur in the short interval corresponding to clamp release, probably concomitantly. In essence, the zigzag array seems designed either to remain as it is or to disassemble in a nearly simultaneous cascade, ideally suited for the synchronous activation of synaptic transmission. How could this be triggered?

The structure suggests an appealingly simple hypothesis. Removing any individual SNAREpin from the zigzag entails breaking contacts with two other SNAREpins, one before and the other after it in the array (SNAREpin 3 in **Fig. 5**). This fracture will almost always break the CPX_{acc} bonds rather than the CPX_{cen} bonds because the former (ΔG = −6.8 kcal mol^{-1}) are much weaker than the latter (ΔG = −9.1 kcal mol^{-1})[21]. When this occurs, the cascade outlined in the last paragraph could be spontaneously triggered (see Discussion below), and the SNAREpins located across the midline of the zigzag would zipper away from each other to form the fusion pore and release neurotransmitter.

If our speculative hypothesis that fusion is triggered by perturbing a single SNAREpin in the array is correct, the activation energy that needs to be provided to enable this triggered fusion will be the energy required to disrupt the two CPX_{acc}–t-SNARE binding sites in which that SNAREpin is engaged, totaling ~14 kcal mol^{-1} (corresponding to ~20 k_BT)[21]. The source of this energy must be from synaptotagmin binding to calcium, the event that triggers release from the clamped state[8]. When synaptotagmin binds calcium, it undergoes a conformational change[28]; as a result, ~21 kcal mol^{-1} of free energy (corresponding to ~33 k_BT) is made available to do work beyond that which is needed for the conformational change itself[29], as the sum of the individual ΔG values for sites 1–3 in C2A and C2B (excluding site 4 because it binds calcium well above the physiological Ca^{2+} concentration range). In addition, the calcium-synaptotagmin complex binds acidic phospholipid-containing bilayers with a K_{eq} of ~2 μM[29], corresponding to ~12 k_BT. The total energy potentially available to perturb the zigzag array when a single synaptotagmin binds its complement of calcium ions is thus ~45 k_BT, greatly exceeding the energy needed to remove its attached SNAREpin (~20 k_BT in our model).

Of course, it is not known how much of Ca^{2+}-synaptotagmin's energy resource is usefully funneled into disrupting the array, but these considerations make it tenable to suggest that activation of a single synaptotagmin molecule (by its bound complement of calcium ions) could be sufficient to dislodge its single bound SNAREpin and that this, in turn, is sufficient to trigger synchronous release of a quantum of neurotransmitter. Twisting, pulling or pushing on a SNAREpin with sufficient (~20 k_BT) energy will remove it from the array, as is illustrated by SNAREpin 3 in **Figure 5**. Though the structural details are still missing, it is easy to imagine how a conformational change in synaptotagmin (perhaps driven by stabilization of its compact

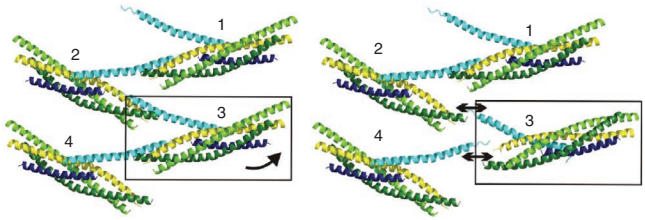

Figure 5 Perturbation of a single SNARE complex in the zigzag array should be sufficient to rapidly disassemble the clamp in response to neuronal stimulus. A small perturbation of one CPX–SNARE complex in the clamped zigzag array would eliminate interactions with both of its neighbors in the array. For example, a disruption of complex 3 would eliminate interaction with complexes 2 and 4. Therefore, if one set of the CPX_{acc}-SNAREpin interactions were to be perturbed by synaptotagmin-Ca^{2+}, then VAMP2 could zipper up, and the entire zigzag array would disassemble very rapidly, releasing the clamp and triggering fusion.

conformation when it binds calcium[28,30,31]) could perturb an attached SNAREpin in this manner. In addition, synaptotagmin is expected to rapidly adhere to the acidic phospholipids (mainly PS and PIP_2) in the nearby synaptic vesicle and/or plasma membrane cytoplasmic leaflets[7,8,32–34], which would also be expected to perturb an attached SNAREpin out of planarity in the array. However, it is important to point out that lipid binding alone (~12 k_BT)[29] does not appear to be sufficient to activate fusion (~20 k_BT) though it may well make an important contribution. The N-terminal domain of CPX (residues 1–25, which precede the CPX_{acc}) also contributes to the activation process *in vivo*[35] but is not needed in our minimal cell-cell fusion system[15]. Also, recent data suggest that Ca^{2+} alone could perturb the CPX–SNARE complexes even in the absence of synaptotagmin, in certain cases[36].

Neurotransmitters can be released within as little as 200 µs after calcium ions enter the nerve terminal[37]. Can activation in our single synaptotagmin-SNAREpin hypothesis keep pace with this? The results from surface force apparatus (SFA) measurements show that the open and closed states in the zigzag array of SNAREpins and CPX (in the absence of synaptotagmin) are separated by an activation energy barrier of ≥ 30 k_BT (ref. 20). Further, the rate of spontaneous fusion from the CPX-clamped state in the flipped SNARE fusion assay, t_{off} ~1 h (ref. 14), indicates an energy barrier also close to 30 k_BT (based on the well-established Kramers-Evans relationship between activation energy and dissociation rate[38]). Starting with the ~30 k_BT value for the spontaneous activation energy barrier from the array, removing a single SNAREpin (by Ca^{2+}-synaptotagmin or any other means) is predicted to reduce the activation energy barrier by ~20 k_BT. The remaining barrier of ~10 k_BT will be transited[38] in 2–20 µs, in no way limiting for the overall time required to release the first quanta of transmitter.

Put differently, the activation energy of ~10 k_BT for unbinding CPX_{acc} in the clamped array implies that single CPX_{acc} dissociation events will occur spontaneously every 2–20 µs, but these will fruitlessly snap back into the array and not result in spontaneous fusion. The activation barrier for spontaneous fusion from the clamped state of ~30 k_BT suggests that it is only when three such contacts are broken (presumably in neighboring SNAREpins) that the fusion pore can successfully open with high probability. In our model, perturbation by Ca^{2+}-synaptotagmin serves to remove two of these three accessory helices, and when the third spontaneously dissociates (2–20 µs), the fusion pore can now open. The lifetime of the Ca^{2+}-synaptotagmin complex, whose lower limit is set by the duration of the rise in local calcium concentration, is far longer than this, so the third and final CPX_{acc} will have many chances to dissociate while calcium is still bound.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

S.S.K., D.T.R. and D.K. did the mutagenesis and protein purification, S.S.K. and D.T.R. carried out the fluorescence measurements, F.L. took the ITC measurements and C.G.G. did the cell-cell fusion assay. L.K., S.B. and J.C. provided technical assistance. Results were analyzed and discussed by all authors. The manuscript was prepared by S.S.K., D.T.R., K.M.R., F.P. and J.E.R.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Plasmid constructs. The constructs used in this study are pET28a oligohistidine-thrombin-syntaxin1A (containing rat Syntaxin1A residues 191–253), pET28a oligohistidine-maltose binding protein (MBP)-thrombin-SNAP25N (containing human SNAP25A (hSNAP25A) residues 7–82 and a C-terminal tryptophan), pET28a oligohistidine-thrombin-SNAP25C (containing hSNAP25A residues 141–203) and pET15b oligohistidine-complexin (containing hComplexin1 residues 1–134 with the following superclamp mutations: D27L, E34F, R37A).The VAMP2 C-terminal truncations were pET28a-oligohistidine-SUMO–VAMP2-60 (hVAMP2 residues 25–60) –VAMP2-65 (hVAMP2 residues 25–65); –VAMP-69 (hVAMP2 residues 25–69); –VAMP-73 (hVAMP2 residues 25–73); –VAMP-77 (hVAMP2 residues 25–77). VAMP-4X and VAMP-3xDA were generated by introducing L70D A74R A81D L84D and D64A D65A D68A mutations, respectively, into pET15b-oligohistidine-thrombin-VAMP2 (human VAMP2 residues 1–96) using a QuikChange mutagenesis kit (Stratagene). VAMP-D64A, VAMP-D65A and VAMP-D68A were also generated in the same fashion. In these constructs, 'thrombin' and 'SUMO' refer to protease cleavage sites.

Protein expression and purification. All constructs were expressed and purified as described previously[21]. Briefly, recombinant fusion proteins were expressed in *Escherichia coli* BL21 (DE3) cells by induction with 1 mM IPTG for 3 h at 37 °C. Cells were harvested and resuspended in breaking buffer (50 mM Tris, 150 mM NaCl, 1 mM tris-(2-carboxyethyl)phosphine (TCEP), pH 7.4) supplemented with protease inhibitors (Sigma), then lysed using a cell disruptor (Avestin). A cleared lysate obtained by centrifugation was incubated for 3–4 h with nickel–nitrilotriacetic acid (Ni-NTA)-agarose (Qiagen) beads. The beads were washed with 25 column volumes of wash buffer (50 mM Tris, 150 mM NaCl, 1 mM TCEP, pH 7.4), followed by 10 column volumes of wash buffer supplemented with 50 mM imidazole. v- and t-SNARE proteins were cleaved with thrombin or SUMO protease (as appropriate for the cleavage site). Complexin was eluted from the beads using 400 mM imidazole in wash buffer, and excess imidazole was removed using a NAP25 desalting column (GE Healthcare). If needed, the proteins were further purified by size-exclusion chromatography (Hi-Load Superdex 75, GE Healthcare).

FRET analysis. Positions Asp193 on SNAP25C and Gln38 on CPX were mutated into cysteines using the Stratagene QuikChange kit. SNAP25 D193C was labeled with the donor probe, stilbene (4-acetamido-4′-((iodoacetyl)amino)-stilbene-2,2′-disulfonic acid, disodium salt, Invitrogen) and CPX Q38C was labeled with the acceptor bimane (monochlorobimane, Invitrogen) as described previously[21]. The double-labeled CPX–SNARE complexes were assembled overnight at 4 °C and purified by gel filtration on a Superdex 75 gel-filtration column. All fluorescence data were obtained on a PerkinElmer LS 55 luminescence spectrometer operating at 25 °C, and the conditions were similar to those used previously[21]. FRET distances were calculated as described previously using a R_0 for the stilbene-bimane FRET pair of 27.5 Å[21].

Toxin accessibility assay. To test the accessibility of VAMP-3xDA to the neurotoxins, 5 μM of either free VAMP-3xDA or CPX–SNARE–3xDA complex was incubated with the neurotoxins at a 1:20 toxin:protein ratio in a Tris buffer, pH 7.4, 150 mM NaCl, containing 100 μM Zn^{2+}, at 37 °C for 2 h, and were analyzed by SDS PAGE–Coomassie blue stain. Botulinum-B and tetanus light chains were purified as described previously[14].

Liposome fusion assay. VAMP2 and t-SNARE proteins were incorporated in liposome at a 1:400 protein:lipid ratio, and liposome fusion assay was carried out as described previously[4,39,40]. Briefly, 45 μl unlabeled t-SNARE liposomes were mixed with 5 μl labeled v-SNARE liposomes in a 96-well plate, and fusion was followed by measuring the increase in 4-nitrobenzo-2-oxa-1,3-diazole (NBD) fluorescence at 538 nm (excitation 460 nm) every 2 min, at 37 °C. At the end of the 2 h reaction, 10 ml of 2.5% dodecylmaltoside was added to the liposomes, and the fusion was plotted as the percentage of the maximal NBD fluorescence[39].

Cell-cell fusion assay. The flipped SNARE cell-cell fusion assay was done essentially as described before[2,14,15,41]. In brief, HeLa cell lines were transiently transfected with flipped VAMP2 (WT or 3xDA) and the red fluorescent protein DsRed2-NES, either with or without CPX and synaptotagmin. After 1 d, transfected v-cells were seeded onto glass coverslips containing cells stably coexpressing flipped syntaxin1, flipped SNAP-25 and CFP-NLS (t-cells). The following day, cells were fixed with 4% paraformaldehyde directly or after treatment with recovery solution (1 U ml^{-1} phosphatidylinositol-specific phospholipase-C (PI-PLC, Molecular Probes), 20 μg ml^{-1} laminin, with or without 1.8 mM EGTA), washed and mounted with ProLong Gold antifade mounting medium (Molecular Probes). Confocal images were acquired on a Zeiss 510-Meta confocal microscope and processed using Adobe Photoshop. Kinetics of the reversal of the CPX clamp by synaptotagmin-Ca^{2+} was essentially carried out as described previously[15], whereby 5 min after the addition of PI-PLC-EGTA, the free Ca^{2+} concentration was raised to 1 mM and the samples were fixed at the noted time intervals. For the calcium sensitivity experiment, free Ca^{2+} was raised to the indicated concentration (ranging from 5 μM to 5,000 μM) and the cells were incubated at 37 °C for 30 min before being fixed for quantitation.

Isothermal titration calorimetry analysis. ITC experiments were carried out as described previously[21]. Typically, ~200 μM of scCPX was titrated into ~10 μM of assembled SNARE complexes in the sample cells, and thermodynamic parameters were calculated using Microcal Origin ITC200 (Mircocal, GE Healthcare), assuming a one-set-of-sites binding model.

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Supplementary Information

A Conformational Switch in Complexin is Required for Synaptotagmin to Trigger Synaptic Fusion

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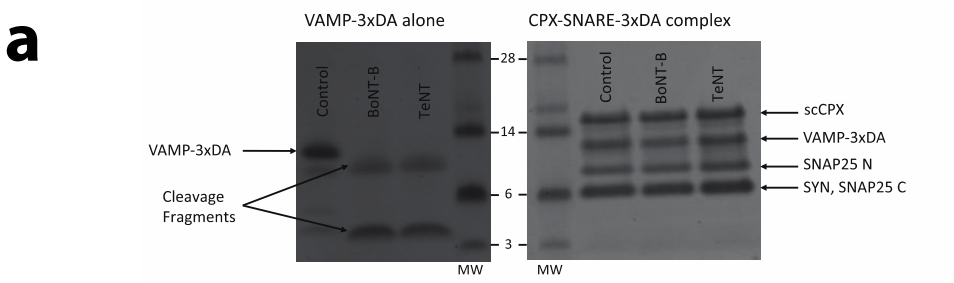

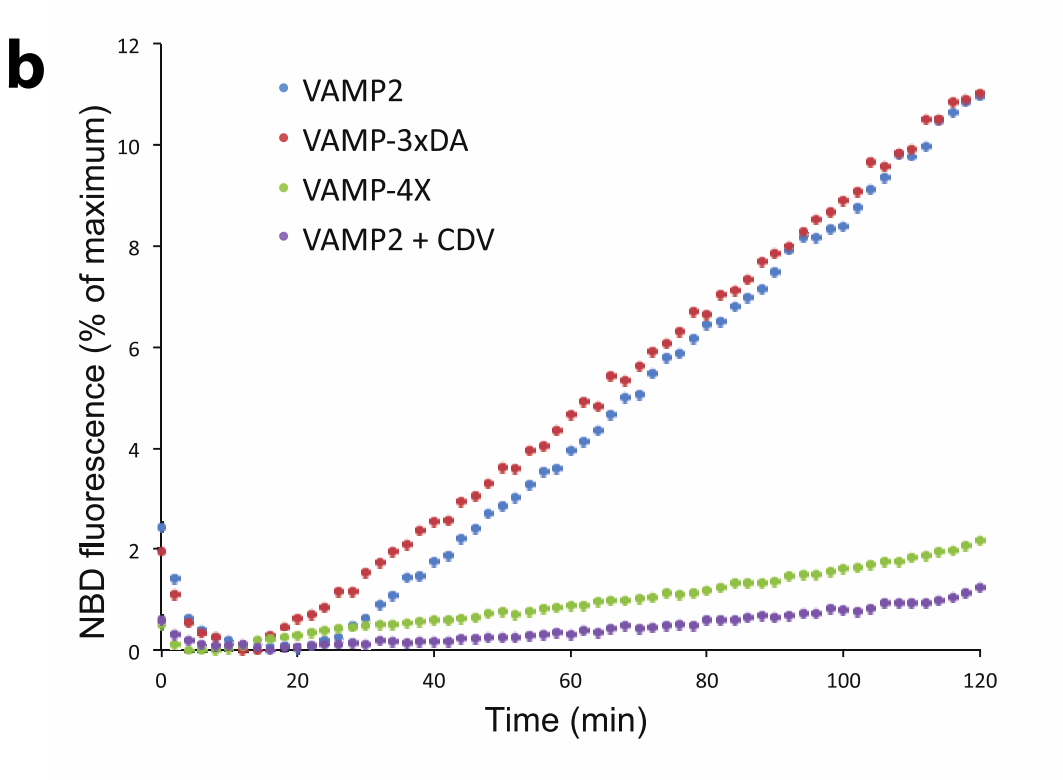

Supplementary Figure 1 Stability and fusion competence of VAMP-3xDA and VAMP-4X Mutants (**a**) VAMP2 is the natural substrate for both Botulism-B (BoNT-B) and Tetanus (TeNT) neurotoxin. Neither toxin can bind nor cleave if the binding residues on VAMP2 (41-45 for TeNT and 63-67 for BoNT-B) are zippered into t-SNARE[1]. The free VAMP-3xDA is readily cleaved by both the neurotoxins (left panel) but is fully protected from cleavage in the presence of t-SNARE and CPX (right panel). This shows that VAMP-3xDA can assemble into a stable CPX-SNARE complex. (**b**) Liposome fusion assay to test the capacity of VAMP-3xDA and VAMP-4X to mediate fusion. The fusion curves for VAMP2 (blue), VAMP-3xDA (red), and VAMP-4X (green) are shown. The cytoplasmic domain of VAMP2 (CDV) was added to the VAMP2 fusion reaction as a negative control (purple).

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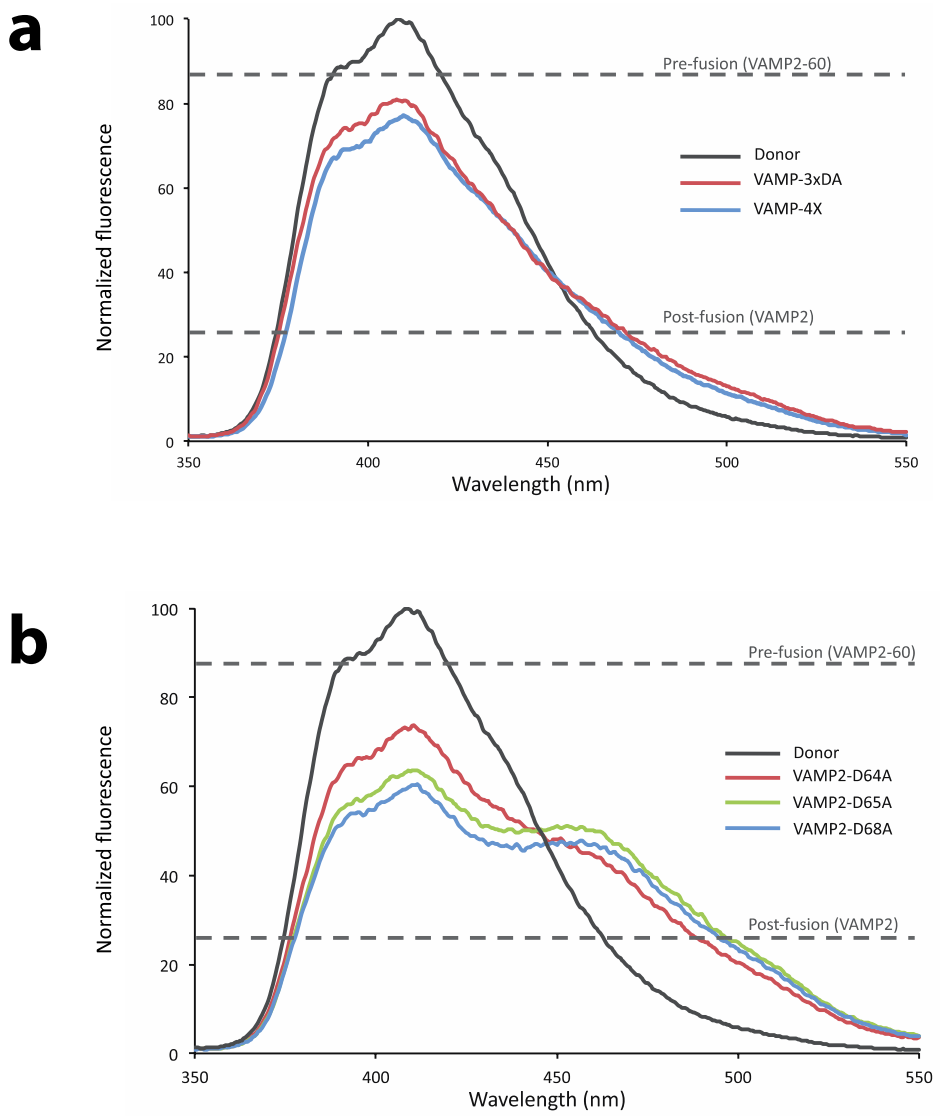
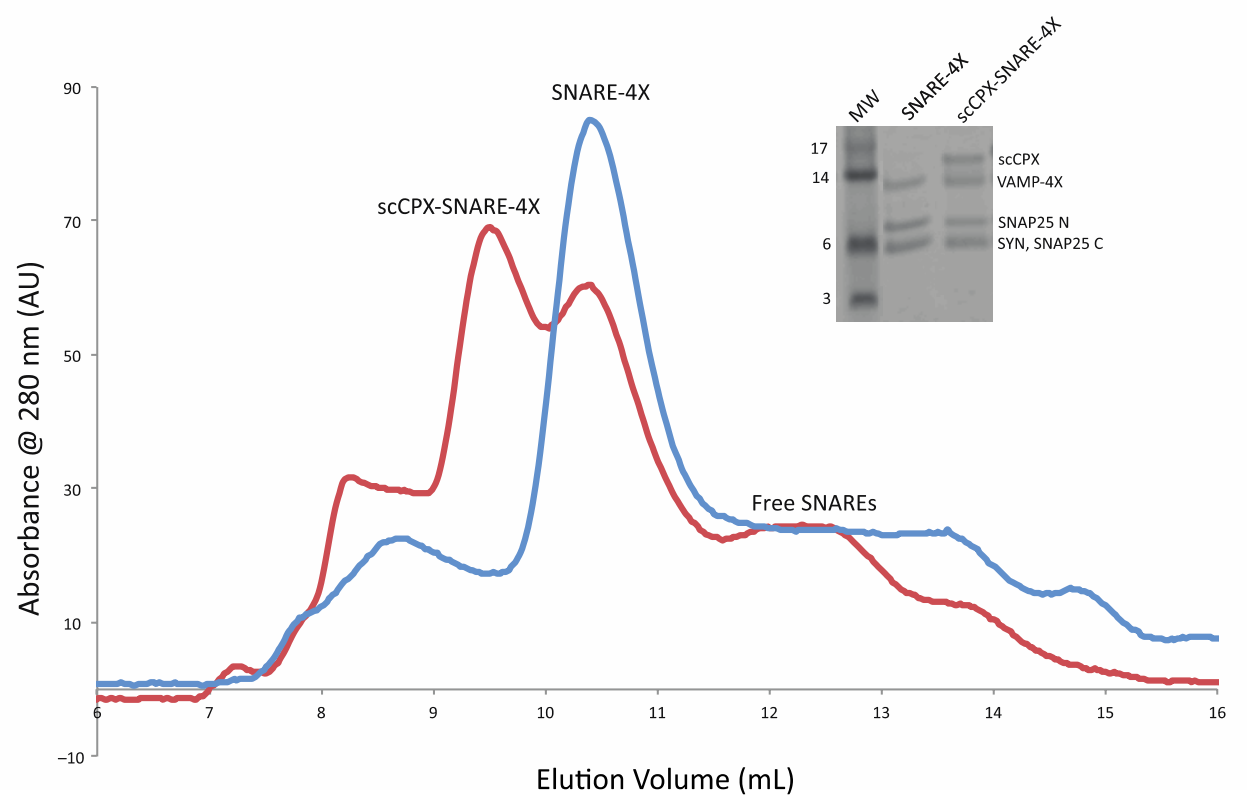
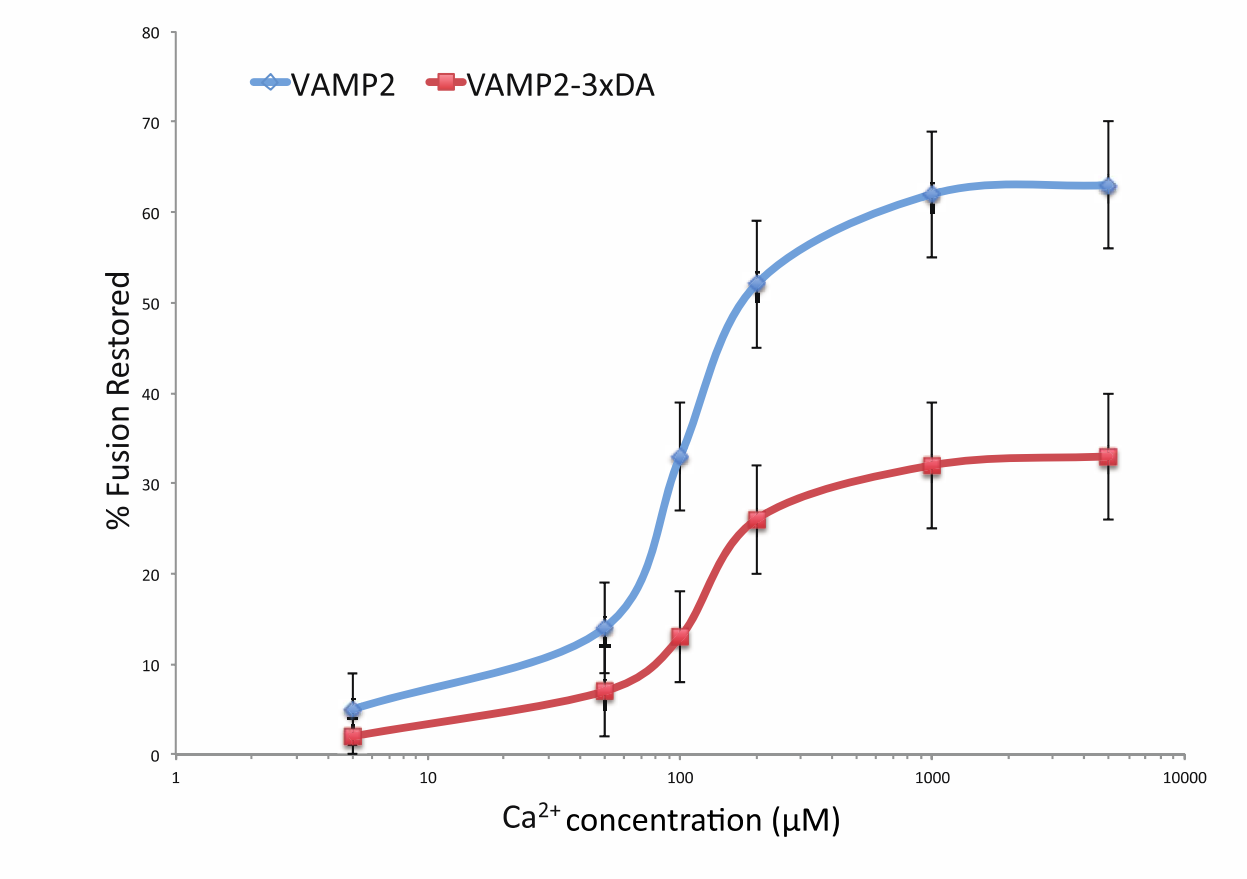


Supplementary Figure 2 Zippering in of all three Asp residues (64, 65, and 68) of VAMP2 required for full switching of the CPX_{acc}. Fluorescence emission spectra of Stilbene-Bimane labeled CPX-SNARE complexes containing (**a**) VAMP-3xDA (VAMP2 D64A, D65A, D68A; red) and VAMP-4X (VAMP2 L70D, A74R, A81D, L84D; blue) and (**b**) VAMP2-D64A (red), VAMP2-D65A (green), and VAMP2-D68A (blue). A representative emission spectrum of Stilbene (Donor)-only CPX-SNARE complex is shown in black. The donor fluorescence level at 410 nm for the pre-fusion (VAMP2-60) and the post-fusion (VAMP2) CPX/SNARE complexes are shown as reference (dashed lines).



Supplementary Figure 3. VAMP4X assembles into stable SNARE complexes with t-SNAREs. A representatitive Superdex 75 elution profile for SNARE4X (blue) and CPX-SNARE4X (red) complex are shown. Inset: Coomassie stained SDS-PAGE gel of the SNARE4X and CPX-SNARE-4X complex peak is shown.



Supplementary Figure 4 Ca^{2+}– Synaptotagmin sensitivity of the VAMP-3xDA mutation. Calcium titration experiment using cells expressing VAMP2 or VAMP3x-DA. 5 min after the addition of PI-PLC/EGTA, the free Ca^{2+} concentration was raised to the indicated concentration (ranging from 5 to 5000µM) and the cells were incubated at 37°C for 30 min. Cells were then fixed and the data quantified as the percentage of the fusion restored.

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