Genetic analysis of the Complexin trans-clamping model for cross-linking SNARE complexes in vivo

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Complexin (Cpx) is a SNARE-binding protein that regulates neurotransmission by clamping spontaneous synaptic vesicle fusion in the absence of Ca^{2+} influx while promoting evoked release in response to an action potential. Previous studies indicated Cpx may cross-link multiple SNARE complexes via a trans interaction to function as a fusion clamp. During Ca^{2+} influx, Cpx is predicted to undergo a conformational switch and collapse onto a single SNARE complex in a cis-binding mode to activate vesicle release. To test this model in vivo, we performed structure–function studies of the Cpx protein in *Drosophila*. Using genetic rescue approaches with *cpx* mutants that disrupt SNARE cross-linking, we find that manipulations that are predicted to block formation of the trans SNARE array disrupt the clamping function of Cpx. Unexpectedly, these same mutants rescue action potential-triggered release, indicating trans–SNARE cross-linking by Cpx is not a prerequisite for triggering evoked fusion. In contrast, mutations that impair Cpx-mediated cis–SNARE interactions that are necessary for transition from an open to closed conformation fail to rescue evoked release defects in *cpx* mutants, although they clamp spontaneous release normally. Our in vivo genetic manipulations support several predictions made by the Cpx cross-linking model, but unexpected results suggest additional mechanisms are likely to exist that regulate Cpx's effects on SNARE-mediated fusion. Our findings also indicate that the inhibitory and activating functions of Cpx are genetically separable, and can be mapped to distinct molecular mechanisms that differentially regulate the SNARE fusion machinery.

synapse | neurotransmitter release | exocytosis

Neurotransmitter release at synapses is a tightly controlled process that is initiated presynaptically within milliseconds of an action potential. Like most cellular fusion events, synaptic vesicle exocytosis is mediated by soluble NSF attachment protein receptors (SNARE) proteins (1). v-SNAREs present on the vesicle (VAMP2/Synaptobrevin) associate with their cognate t-SNAREs on the target plasma membrane (SNAP25 and Syntaxin 1) to form a coiled-coil four-helix SNAREpin bundle that drives membrane fusion (2, 3). During vesicle fusion, the v-SNARE Synaptobrevin is predicted to zipper onto a preassembled t-SNARE dimer to form the fully assembled SNARE complex, bringing the synaptic vesicle closer to the plasma membrane (4, 5). This process is characterized by a sequential SNARE folding pathway that includes a half-zipped intermediate SNARE complex (6). It is this intermediate SNARE complex that may be the key control point for regulating fusion, with proteins that inhibit or promote zippering serving as clamps or activators of the release process, respectively. Indeed, several neuronal specific SNARE-binding proteins have emerged as key regulators of the SNARE fusion machine (7, 8). The synaptic vesicle protein Synaptotagmin (Syt) serves as a Ca^{2+} sensor that stimulates synaptic vesicle fusion in response to action potential triggered Ca^{2+} influx (9, 10). In addition to Syt, the cytosolic protein Complexin (Cpx) has emerged as an important cofactor for controlling synaptic vesicle

fusion, potentially through its ability to regulate SNARE zippering (11, 12).

Cpx is a cytosolic α -helical protein that binds to SNARE complexes (13). Initial in vitro studies using cell–cell fusion or lipid-mixing assays indicated that Cpx prevents in vitro membrane fusion events, suggesting Cpx may act as a fusion clamp to prevent premature exocytosis in the absence of Ca^{2+} (11, 14, 15). Consistent with the role of Cpx in clamping synaptic fusion, in vivo experiments in *Drosophila* revealed a dramatic increase in spontaneous synaptic vesicle fusion events (minis) in *cpx* null mutants (16). In addition to enhanced minis, *cpx* mutants have reduced evoked release. Together with data from other genetic systems (17–21), an emerging model is that Cpx plays a dual role in release, acting as a vesicle fusion clamp that prevents spurious fusion of vesicles at some synapses, while simultaneously promoting synchronous release in response to an action potential at all synapses (16, 17, 19–22). Structurally, these dual activities are likely to require an α -helix motif found within Cpx that is divided into an accessory and central helix region (Fig. 1A). Binding of the Cpx central helix to the SNARE complex is absolutely required for both clamping and stimulating properties (17, 23). Outside the central helix, the N terminus and the accessory helix has been postulated to contribute to the stimulatory and inhibitory properties of Cpx (22). How these distinct domains are coordinated to regulate spontaneous and evoked release, and whether these activities are required sequentially or in parallel, is poorly understood.

Studies using a C-terminal truncated v-SNARE protein to mimic partial SNARE zippering, together with “superclamp”

Significance

Synaptic vesicle fusion at synapses is the primary mechanism by which neurons communicate. A highly conserved membrane fusion machine known as the SNARE complex mediates this process. In addition, neuronal SNARE-binding regulatory proteins have evolved to control the kinetics and speed of SNARE assembly at synapses. One such SNARE-binding protein, Complexin, has been found to inhibit synaptic vesicle fusion in the absence of an action potential and activate SNARE-mediated release during stimulation. Here we examine molecular models for how Complexin can mediate these unique effects on the SNARE fusion machine using genetic rescue experiments in *Drosophila*. We find that alternate SNARE-binding mechanisms by Complexin are likely to contribute to distinct inhibitory and activating functions in vivo.

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Fig. 1. The clamping and activation properties of Cpx are proposed to be mediated by a trans Cpx/SNARE array that forms between the accessory/central domains of Cpx and partially zippered prefusion SNARE complexes. (A) Structure of Cpx, and the Cpx mutants used in this study. (B) The trans Cpx/SNARE array (zigzag array) that is proposed to form between Cpx and SNARE complexes. A conformational switch in Cpx from an open (trans-SNARE) to a closed (cis-SNARE) state is proposed to mediate Ca²⁺-dependent triggering of vesicle fusion. The positions of mutations used in this study are marked with colors matching Fig. 1A. (C) Hydrophobic residues that mediate the interaction of the Cpx accessory helix with t-SNAREs of a partially zippered SNARE complex are conserved in both mCpx and DmCpx. Space fill model of the accessory domain of mCpx and DmCpx is shown. Blue and gray residues represent the interface between the Cpx accessory domain and t-SNAREs. Conserved hydrophobic residues are illustrated in gray. (D) Both mCpx and DmCpx exhibit clamping properties in in vitro cell-cell fusion assays. (E) SB mCpx exhibits clamping properties similar to WT mCpx in cell-cell fusion assays. (F) SB mCpx exhibits decreased Syt-stimulated cell-cell fusion compared with WT mCpx. Error bars are SEM, and horizontal lines indicate statistical comparisons determined using one-way ANOVA with post hoc Tukey analysis for D–F.

mutations in the Cpx accessory helix that enhance its inhibitory activity (24), have suggested a structural basis by which Cpx might regulate these dual activities (25–27). Crystallization of the Cpx-bound truncated SNARE complex revealed that the N-terminal accessory helix domain of Cpx extends away from its central helix domain at a 45° angle to form a trans interaction with a second SNARE complex, binding within the open t-SNARE pocket of the partially zippered SNAREs (trans Cpx/SNARE or zigzag array). This finding suggested a structural basis by which Cpx clamps vesicles in a fusion capable state (26). In response to Ca²⁺, the trans Cpx/SNARE array is predicted to collapse, with the Cpx helix undergoing a conformational switch to associate with the fully zippered cis-SNARE complex, providing a switch mechanism by which Cpx might promote release (Fig. 1B) (25). However, the in vivo relevance of such a mechanism in neurotransmitter release is unknown. Here, we undertook a genetic

approach to test the trans Cpx/SNARE array and switch-release model of Cpx using the *Drosophila melanogaster* (Dm) neuromuscular junction (NMJ) as a model synapse.

Results

Drosophila has a single Cpx homolog that is enriched in pre-synaptic nerve terminals. Electrophysiological analysis at NMJs in *cpx* null mutants revealed dramatically enhanced spontaneous release and reduced evoked release compared with controls (16), consistent with Cpx's in vitro properties (14, 15, 26). Based on the crystal structure of the Cpx/SNARE array, we designed a series of mutations that were previously shown to modulate Cpx function as a vesicle clamp in in vitro cell-cell fusion assays (Fig. 1A and B). A key molecular determinant of Cpx's ability to bridge two SNARE complexes in vitro is the presence of hydrophobic residues in the accessory helix that bind to a second partially zippered t-SNARE complex to form the trans Cpx/SNARE array. Hydrophobic mutations in this region [termed superclamp (SC)] increased the ability of mammalian (m) Cpx to promote clamping in in vitro cell-cell “flipped” fusion assays, whereas charged residue substitutions within this region [termed nonclamp (NC)] reduced clamping (26). Furthermore, mutations that disrupt the continuity of the Cpx helix [termed helixbreaker (HB)] abolished clamping by Cpx in this assay (26).

To determine if a similar trans Cpx/SNARE array interaction might occur in *Drosophila*, we compared the accessory helix of *Drosophila* Cpx (DmCpx) to that of mCpx. The DmCpx accessory helix contains hydrophobic residues (A35, I46, and A49) that are predicted to orient and align similarly to the hydrophobic residues found in mCpx (Fig. 1C). We found that WT DmCpx clamps cell-cell fusion in vitro similar to WT mCpx (Fig. 1D) [WT DmCpx = $7.1 \pm 1.6\%$ ($n = 3$) vs. WT mCpx = $5.0 \pm 1.4\%$ ($n = 4$); $P > 0.05$], suggesting a trans Cpx/SNARE array interface may be conserved in *Drosophila*.

In addition to clamping release to prevent spontaneous fusion, Cpx also promotes Syt-dependent vesicle fusion (16, 17, 19–22). Ca²⁺-dependent triggering of release has been suggested to require a conformational switch in Cpx from an open (Cpx extends away from the SNARE surface at a ~45°) to a closed (Cpx lies on the surface of a postfusion SNARE complex) state (Fig. 1B) (26). This switch is mediated by hydrogen bond and salt bridge interactions of residues in VAMP2 (D64, D65, D68) with residues within the Cpx central helix (R48, Y52) (25, 28). The in vitro relevance of this interaction was tested previously using mutations within VAMP2 (25). Here, we designed an equivalent Cpx mutant [R48D/E, Y52A, termed switchbreaker (SB)] that is predicted to reduce Cpx's ability to switch from an open to closed conformation, and examined both the clamping and stimulating properties of SB mCpx using in vitro cell-cell fusion assays. Despite mutations in its central helix, SB mCpx maintained its ability to clamp in in vitro cell-cell fusion similar to WT mCpx (Fig. 1E) [SB mCpx = $7.6 \pm 0.8\%$ ($n = 3$) vs. WT mCpx = $5.0 \pm 0.7\%$ ($n = 4$); $P > 0.05$]. In contrast, activation of cell-cell fusion with SB mCpx was significantly reduced following addition of Ca²⁺ and Syt (Fig. 1F) [SB mCpx = $1.5 \pm 0.4\%$ fold increase ($n = 3$) vs. WT mCpx = 3.2 ± 0.5 fold increase ($n = 4$); $P < 0.05$]. These data indicate that interactions involving mCpx residues R48 and Y52 with zippering or fully assembled SNAREs may be important to trigger release, but are not required for Cpx to clamp fusion.

To examine the in vivo relevance of the Cpx trans array clamping/switch model, we generated transgenic *Drosophila* strains expressing a series of mCpx mutants that are predicted to disrupt these activities. First, we tested the requirement of the interaction between the accessory domain of Cpx and the half-zippered t-SNARE complex using the SC and NC mCpx mutants. Using in vitro experiments, these mutants exhibited enhanced and reduced ability, respectively, to bind and clamp compared with wild-type Cpx

Table 1. Thermodynamic parameters of various mCpx binding to postfusion, cis-DmSNARE complexes

Interaction	Stoichiometric coefficient, <i>N</i>	<i>K_D</i> , nM	ΔH , kcal·mol ⁻¹	ΔS , cal·mol ⁻¹ ·°C ⁻¹	ΔG , kcal·mol ⁻¹	ΔG , <i>k_BT</i>
WT mCpx/postfusion DmSNARE	0.97 ± 0.03	33 ± 2	-27.2 ± 0.1	-57.6 ± 0.5	-10.1 ± 0.1	17.2 ± 0.1
SB mCpx/postfusion DmSNARE	1.01 ± 0.02	1104 ± 95	-7.7 ± 0.2	1.1 ± 0.7	-8.1 ± 0.1	13.7 ± 0.1

DmSNAREs is 33 ± 2 nM, similar to the affinity constant of mCpx with mammalian cis SNAREs (25). Compared with WT mCpx, the SB mCpx mutant displayed a weaker affinity (~1.1 ± 0.1 μM). Additionally, the binding enthalpy of SB mCpx with cis DmSNAREs was weaker compared with WT mCpx by ~20 kcal·mol⁻¹ (SB mCpx: $\Delta H = -7.7 \pm 0.2$ kcal·mol⁻¹ vs. WT mCpx: $\Delta H = -27.2 \pm 0.1$; Table 1). These differences are similar to those previously reported using the VAMP2 SB mutant (D64A, D65A, and D68A) designed to disrupt hydrogen bond and salt bridge interactions with the Cpx central helix (R48 and Y52). Despite the impaired association of SB mCpx with fully assembled SNARE complexes and the inability to rescue evoked release, SB mCpx was able to rescue minis similarly to WT mCpx [SB mCpx rescues = 16.8 ± 1.3 Hz (*n* = 11) vs. WT mCpx rescue = 14.6 ± 1.1 Hz (*n* = 18); *P* > 0.05] (Fig. 2). These data indicate the central helix is sufficient to support evoked release, whereas the accessory helix is needed to maintain its clamp function. In summary, our findings suggest that Cpx promotes Ca²⁺-dependent vesicle fusion by mechanisms that are independent of accessory domain interactions, and instead require residues within the central helix of Cpx. The independent effects on spontaneous vs. evoked release in both the SB and trans interaction-abolishing mutants indicate the Cpx clamping and promoting functions are genetically separable, consistent with prior studies (17, 19, 20, 31–33).

Discussion

To characterize the mechanisms by which Cpx regulates synaptic vesicle fusion, we performed an *in vivo* structure–function study by expressing WT and mutant Cpx transgenes in *cpx* null mutants. Unexpectedly, Cpx mutants that disrupt the formation of a trans Cpx/SNARE array, as confirmed by *in vitro* binding experiments, were capable of promoting action potential-triggered release. However, these same mutants failed to rescue the clamping defect in *cpx*, indicating differential effects on Cpx's two primary roles in exocytosis. In contrast, mutants that altered Cpx interactions with the fully zipper SNARE complex through cis rather than trans interactions, failed to promote evoked vesicle release, but clamped spontaneous fusion normally. These findings demonstrate that the clamping and promoting mechanism of Cpx are genetically separable, and may be regulated by distinct mechanisms at the level of SNARE interactions.

Consistent with previous *in vitro* experiments (26), mutants that disrupted the ability of Cpx to form a rigid bridge between two SNARE complexes failed to support a clamping function at the synapse *in vivo* (see HB mCpx rescues in Fig. 2). The Cpx N terminus (1–48) was absolutely required to clamp spontaneous fusion *in vivo* (mCpx 51–134; Fig. 2), indicating Cpx may form a cross-linked SNARE structure *in vivo*. Surprisingly however, NC Cpx mutants that disrupted the interaction between Cpx accessory domain and the t-SNAREs of a second SNARE complex were able to largely rescue the elevated spontaneous fusion rate found in *cpx* null mutants (Fig. 2). This observation was not consistent with the cross-linked SNARE model given that NC mCpx exhibited reduced clamping activity in previous cell–cell fusion assays (26), and that ITC experiments demonstrated that NC mCpx does not associate with either mammalian or *Drosophila* SNAREs *in vitro* (Fig. S3 and Table S2). The NC mCpx mutant analysis raises the question of whether trans interactions are essential for clamping *in vivo*. It is possible that association of the Cpx N terminus with a second prefusion

SNARE complex may be more difficult to perturb *in vivo* than *in vitro*. For example, additional factors might stabilize this interaction, or secondary residues flanking the primary association site in the t-SNARE complex may contribute to the Cpx/t-SNARE association *in vivo*. The inability of the HB mCpx and truncated mCpx 51–134 to rescue the elevated mini rate indicates a rigid N-terminal extension away from the central SNARE-binding domain is important for Cpx-mediated vesicle clamping. However, we cannot rule out that these mutants may also disrupt a distinct *in vivo* Cpx–SNARE interaction beyond the predicted trans Cpx/SNARE array. Indeed, there is evidence that mutations within the N-terminal domain of Cpx may alter release by blocking cis–SNARE zipper as well (34). Unlike NC mCpx mutants, we observed a strong effect of the SC mCpx mutants, which enhanced the clamping function of Cpx *in vivo*, and enhanced formation of trans interactions *in vitro* (26).

In contrast to the effects of Cpx cross-linking mutants on their clamping properties, these same mutations were capable of rescuing action potential-triggered synaptic vesicle fusion. Indeed, even an N-terminal truncation mutant completely lacking the Cpx accessory helix was capable of restoring evoked vesicle release *in vivo* (Fig. 3). Although we cannot exclude the possibility that these mutants exert subtle effects on vesicle pool sizes, or vesicle release kinetics and synchronicity (19, 21, 31, 35), our findings indicate a trans Cpx/SNARE array is not absolutely required for vesicle release following an action potential.

The SB mutant that alters Cpx interactions with the fully zipper SNARE complex *in cis*, rather than with a second SNARE complex *in trans*, failed to promote evoked vesicle release efficiently (Fig. 3). Why does this SB mCpx mutant fail to rescue evoked release? The crystal structure of wild-type Cpx with the postfusion SNARE complex shows that R48 and Y52 interact with D64, D65, and D68 in the C terminus of VAMP2 (28). ITC measurements show that the SB mutations in Cpx result in a loss of ~ -20 kcal·mol⁻¹ in binding enthalpy to the postfusion SNARE complex (Table 1 and Fig. 4), which is similar to the enthalpy loss caused by the D64A, D65A, and D68A mutations in VAMP2 (25), indicating similar defects to those found in the SB mCpx mutants. FRET studies demonstrated that Cpx remains in the open state (Cpx extends away from postfusion SNARE surface at ~45°) when D64, D65, and D68 are mutated (25). Hence, the R48, Y52 mutation of Cpx is likely to abolish the transition of Cpx from an open to closed state as the molecular driving force of this transition is impaired. Given the N-terminal domain of Cpx (1–50) is not required for evoked release (Fig. 3), it is also possible that a conformational transition of the Cpx central helix from an open to closed state is sufficient for evoked release. Interestingly, the SB mCpx mutant clamps spontaneous release normally (Fig. 2); this is the first Cpx mutant that selectively alters the ability of Cpx to promote evoked vesicle fusion, while leaving the clamping function intact.

In summary, these results indicate that the clamping and activating functions of Cpx are genetically separable, and are regulated through distinct SNARE interaction mechanisms. This conclusion is consistent with additional studies examining the contribution of the Cpx C terminus in regulating synaptic vesicle release (17, 31–33, 36). What mechanism is ultimately used to separate Cpx's role in spontaneous vs. evoked release is still unclear, and may involve a complex interplay with the SNARE complex and other SNARE-associated proteins, including Syt (37, 38). These distinct roles may also be manifest through

effects of Cpx on molecularly distinct pools of vesicles (39), or on specific active zones dedicated to evoked vs. spontaneous release (40). Given that Cpx mutants that interrupt the trans Cpx/SNARE array support evoked release, it is unlikely that a Cpx-mediated cross-linking of SNARE complexes is required during Ca^{2+} -triggered release. As such, the SB Cpx mutant may impede the transition of the Cpx central helix from an open to closed conformation with a single zipper SNARE complex. Within Cpx, therefore, the central α -helix is the primary domain that associates with SNARE complexes (28), and is absolutely required for all Cpx function (17, 20, 23). The N-terminal accessory domain is necessary for clamping functions, as demonstrated in this study and others (23, 30, 41). The nonhelical N-terminal domain of Cpx is required for promotion of evoked release at some synapses (18, 30), whereas the C terminus functions to regulate both clamping and priming through its ability to target Cpx to membranes (17, 32, 33, 36, 42). How these distinct domains work in concert to regulate the overall mode of synaptic release is an important question, and likely to be critical for distinct plasticity mechanisms that alter release through changes in Cpx function (17, 18, 30, 33). It is also possible that clamping and promotion of vesicle release may be mechanistically coupled through currently unknown pathways.

Materials and Methods

Protein Constructs, Expression, and Purification. Recombinant proteins were expressed and purified using standard bacterial expression systems. See *SI Materials and Methods* for details.

Isothermal Titration Calorimetry Analysis. ITC experiments were performed as previously described (26). See *SI Materials and Methods* for details.

Cell–Cell Fusion Assays. Cell–cell fusion assays were performed as previously described (14, 24). See *SI Materials and Methods* for details.

Drosophila Genetics. *Drosophila melanogaster* were cultured on standard medium at 25 °C. *Drosophila* transgenic lines were generated using standard methods. Briefly, WT and mutant mCpx were transgenes were recombined into *cpx^{SH1}* null mutant (16) and expressed using neuronal C155 elav-Gal4 driver. See *SI Materials and Methods* for details.

Electrophysiology. Muscle fiber 6 (segment A3) of dissected third instar larvae was used for all sharp electrode potential recordings as previously described (16). See *SI Materials and Methods* for details.

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- Söllner T, et al. (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362(6418):318–324.
- Weber T, et al. (1998) SNAREpins: Minimal machinery for membrane fusion. *Cell* 92(6):759–772.
- Sutton RB, Fasshauer D, Jahn R, Brunger AT (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* 395(6700):347–353.
- Li F, et al. (2007) Energetics and dynamics of SNAREpin folding across lipid bilayers. *Nat Struct Mol Biol* 14(10):890–896.
- Rizo J, Rosenmund C (2008) Synaptic vesicle fusion. *Nat Struct Mol Biol* 15(7):665–674.
- Li F, et al. (2014) A half-zipped SNARE complex represents a functional intermediate in membrane fusion. *J Am Chem Soc* 136(9):3456–3464.
- Südhof TC, Rothman JE (2009) Membrane fusion: Grappling with SNARE and SM proteins. *Science* 323(5913):474–477.
- Südhof TC (2013) Neurotransmitter release: The last millisecond in the life of a synaptic vesicle. *Neuron* 80(3):675–690.
- Littleton JT, Stern M, Schulze K, Perin M, Bellen HJ (1993) Mutational analysis of *Drosophila* synaptotagmin demonstrates its essential role in Ca^{2+} -activated neurotransmitter release. *Cell* 74(6):1125–1134.
- Geppert M, et al. (1994) Synaptotagmin I: A major Ca^{2+} sensor for transmitter release at a central synapse. *Cell* 79(4):717–727.
- Malsam J, et al. (2012) Complexin arrests a pool of docked vesicles for fast Ca^{2+} -dependent release. *EMBO J* 31(15):3270–3281.
- Bharat TA, et al. (2014) SNARE and regulatory proteins induce local membrane protrusions to prime docked vesicles for fast calcium-triggered fusion. *EMBO Rep* 15(3):308–314.
- McMahon HT, Missler M, Li C, Südhof TC (1995) Complexins: Cytosolic proteins that regulate SNAP receptor function. *Cell* 83(1):111–119.
- Giraudo CG, Eng WS, Melia TJ, Rothman JE (2006) A clamping mechanism involved in SNARE-dependent exocytosis. *Science* 313(5787):676–680.
- Schaub JR, Lu X, Doneske B, Shin YK, McNew JA (2006) Hemifusion arrest by complexin is relieved by Ca^{2+} -synaptotagmin I. *Nat Struct Mol Biol* 13(8):748–750.
- Huntwork S, Littleton JT (2007) A complexin fusion clamp regulates spontaneous neurotransmitter release and synaptic growth. *Nat Neurosci* 10(10):1235–1237.
- Cho RW, Song Y, Littleton JT (2010) Comparative analysis of *Drosophila* and mammalian complexins as fusion clamps and facilitators of neurotransmitter release. *Mol Cell Neurosci* 45(4):389–397.
- Maximov A, Tang J, Yang X, Pang ZP, Südhof TC (2009) Complexin controls the force transfer from SNARE complexes to membranes in fusion. *Science* 323(5913):516–521.
- Hobson RJ, Liu Q, Watanabe S, Jorgensen EM (2011) Complexin maintains vesicles in the primed state in *C. elegans*. *Curr Biol* 21(2):106–113.
- Martin JA, Hu Z, Fenz KM, Fernandez J, Dittman JS (2011) Complexin has opposite effects on two modes of synaptic vesicle fusion. *Curr Biol* 21(2):97–105.
- Jorquera RA, Huntwork-Rodriguez S, Akbergenova Y, Cho RW, Littleton JT (2012) Complexin controls spontaneous and evoked neurotransmitter release by regulating the timing and properties of synaptotagmin activity. *J Neurosci* 32(50):18234–18245.
- Xue M, et al. (2009) Tilting the balance between facilitatory and inhibitory functions of mammalian and *Drosophila* Complexins orchestrates synaptic vesicle exocytosis. *Neuron* 64(3):367–380.
- Xue M, et al. (2010) Binding of the complexin N terminus to the SNARE complex potentiates synaptic-vesicle fusogenicity. *Nat Struct Mol Biol* 17(5):568–575.
- Giraudo CG, et al. (2009) Alternative zippering as an on-off switch for SNARE-mediated fusion. *Science* 323(5913):512–516.
- Krishnakumar SS, et al. (2011) A conformational switch in complexin is required for synaptotagmin to trigger synaptic fusion. *Nat Struct Mol Biol* 18(8):934–940.
- Kümmel D, et al. (2011) Complexin cross-links prefusion SNAREs into a zigzag array. *Nat Struct Mol Biol* 18(8):927–933.
- Li F, et al. (2011) Complexin activates and clamps SNAREpins by a common mechanism involving an intermediate energetic state. *Nat Struct Mol Biol* 18(8):941–946.
- Chen X, et al. (2002) Three-dimensional structure of the complexin/SNARE complex. *Neuron* 33(3):397–409.
- Reim K, et al. (2001) Complexins regulate a late step in Ca^{2+} -dependent neurotransmitter release. *Cell* 104(1):71–81.
- Xue M, et al. (2007) Distinct domains of complexin I differentially regulate neurotransmitter release. *Nat Struct Mol Biol* 14(10):949–958.
- Iyer J, Wahlmark CJ, Kuser-Ahnert GA, Kawasaki F (2013) Molecular mechanisms of COMPLEXIN fusion clamp function in synaptic exocytosis revealed in a new *Drosophila* mutant. *Mol Cell Neurosci* 56:244–254.
- Wragg RT, et al. (2013) Synaptic vesicles position complexin to block spontaneous fusion. *Neuron* 77(2):323–334.
- Kaesler-Woo YJ, Yang X, Südhof TC (2012) C-terminal complexin sequence is selectively required for clamping and priming but not for Ca^{2+} triggering of synaptic exocytosis. *J Neurosci* 32(8):2877–2885.
- Bykhovskaia M, Jagota A, Gonzalez A, Vasin A, Littleton JT (2013) Interaction of the complexin accessory helix with the C-terminus of the SNARE complex: Molecular-dynamics model of the fusion clamp. *Biophys J* 105(3):679–690.
- Dhara M, et al. (2014) Complexin synchronizes primed vesicle exocytosis and regulates fusion pore dynamics. *J Cell Biol* 204(7):1123–1140.
- Buhl LK, et al. (2013) Differential regulation of evoked and spontaneous neurotransmitter release by C-terminal modifications of complexin. *Mol Cell Neurosci* 52:161–172.
- Ramirez DM, Kavalali ET (2012) The role of non-canonical SNAREs in synaptic vesicle recycling. *Cell Logist* 2(1):20–27.
- Xu J, Brewer KD, Perez-Castillejos R, Rizo J (2013) Subtle interplay between synaptotagmin and complexin binding to the SNARE complex. *J Mol Biol* 425(18):3461–3475.
- Kavalali ET, et al. (2011) Spontaneous neurotransmission: An independent pathway for neuronal signaling? *Physiology (Bethesda)* 26(1):45–53.
- Melom JE, Akbergenova Y, Gavornik JP, Littleton JT (2013) Spontaneous and evoked release are independently regulated at individual active zones. *J Neurosci* 33(44):17253–17263.
- Yang X, Kaesler-Woo YJ, Pang ZP, Xu W, Südhof TC (2010) Complexin clamps asynchronous release by blocking a secondary Ca^{2+} sensor via its accessory α helix. *Neuron* 68(5):907–920.
- Seiler F, Malsam J, Krause JM, Söllner TH (2009) A role of complexin-lipid interactions in membrane fusion. *FEBS Lett* 583(14):2343–2348.