Complexin: Does It Deserve Its Name?

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Knockout and other perturbations of complexins have provided important insights and elicited controversies about their role in neurotransmitter release. New work by Yang et al. in this issue of *Neuron* adds important detail and complexity to existing concepts—particularly on the nature of a Ca^{2+}-dependent complexin-synaptotagmin switch for the triggering of exocytosis. But it also provokes thoughts about alternative interpretations, which might result in a simpler model of complexin function.

Complexins (Cpxs) are relatively small synaptic proteins with molecular weights of about 16 kD. They bind reversibly to the so-called SNARE complex and, thereby, become part of the synaptic release apparatus, which makes vesicles fuse with the plasma membrane. Knockout (KO) studies and mutational analyses have resulted in divergent views about the functional role of Cpxs. Perturbations of the proteins cause an increase in spontaneous and asynchronous release of neurotransmitter in some types of synapses studied, indicating an inhibitory role of the intact proteins (see below). On the other hand, many of such studies also showed that the highly synchronous release during action potential stimulation was severely compromised (see Brose, 2008, for review). Yang et al. (2010) employ a knockdown and rescue approach to shed new light on some of the "complex" actions of Cpx. In this Preview I will try to highlight some of the new findings in the framework of current concepts about Cpx function and will make an attempt to view these molecules from a somewhat different angle.

The SNARE complex is the core of the synaptic release machinery, consisting of the three proteins syntaxin (Syx), synaptobrevin (Syb), and SNAP25 (Sudhof and Rothman, 2009). One of these, Syx, is anchored in the plasma membrane, while Syb is anchored in the vesicle membrane. Together with SNAP25 they form a four-helix bundle (SNAP25 contributing two helices) that pulls the two membranes together when helices associate with each other or "zipper up." Cpxs bind to a groove between two helices in the complex, thereby stabilizing it (reviewed by Brose, 2008). The fifth player is the Ca^{2+}-binding protein synaptotagmin (Syt), which is generally held to be the "Ca^{2+} sensor" of the release machinery because of its Ca^{2+}-binding properties. Much of the early work on Cpx centered around the following questions: what is the role of Cpx (stabilizing the complex or preventing initiation and/or completion of zippering); does Cpx compete with Syt for binding or do these proteins stabilize each other; what is the sequence of binding?

As already mentioned, many perturbations of Cpx function cause an increase in spontaneous release, which led to the proposal that Cpxs act as fusion clamps that prevent premature exocytosis. However, clamping release clearly cannot be the only action of Cpx in view of multiple indications of a positive role of Cpx in Ca^{2+}-triggered synchronous release (Brose, 2008). Also, both spontaneous and triggered release in hippocampal neurons were found to be decreased upon knocking out all Cpx isoforms expressed in brain (Brose, 2008). An explanation for the dual effect of Cpxs was first provided in a structure function analysis by Xue et al. (2007), who showed that the very N terminus of murine Cpx is essential for a facilitatory function, whereas the adjacent accessory helix exerts a mild inhibitory action. The location of the Cpx N terminus near the C-terminal end of the SNARE complex (i.e., near the membrane anchors of Syb and Stx), together with the demonstration of an interaction between the two entities in vitro led Xue et al. (2007, 2010) to the conclusion that this interaction promotes fusogenicity of the SNARE complex by stabilizing its C terminus (see Figure 1 for a schematic representation of Cpx domains). Similarly, analyses of N-terminal deletion mutants led to the conclusion that Cpxs control the force transfer from SNARE complex to fusing membranes (Maximov et al., 2009). Several investigations also agreed on the point, that the "central helix" of Cpx, which binds to the SNARE complex, is essential for its function (Brose, 2008).

Controversy, however, remained regarding the nature of the interaction between Cpxs and Syts. Tang et al. (2006) had proposed the so-called Cpx/Syt switch model (Cpx-Syt-switch), based on in vitro findings that Syt 1 competes with Cpx for SNARE binding. According to this concept, Cpx clamps the release apparatus in a metastable but inactive state, thereby preventing spontaneous release. The model posits that Cpx is displaced by Syt upon Ca^{2+} binding, triggering fast release. A more specific mechanism for the clamping action of Cpx was subsequently proposed (Sudhof and Rothman, 2009), based on some sequence similarly between the accessory helix of Cpx and the membrane-proximal part of the syt SNARE motif, as well as on mutagenesis experiments. According to this concept, the accessory helix of Cpx binds to the partially zippered SNARE complex and thereby competes with Syb, stalling it in a partially zippered state. The Cpx-Syt-switch model, however, is not in line with data, which indicate that Cpx and Syt can bind simultaneously to SNARE complexes (reviewed by Sørensen, 2009).

In the most recent work Yang et al. (2010) performed a number of knockdown (KD) and rescue experiments to test and corroborate the Cpx-Syt-switch model, which, however, also led to some modifications of the model. First, they confirmed the KD results of Maximov et al. (2009),

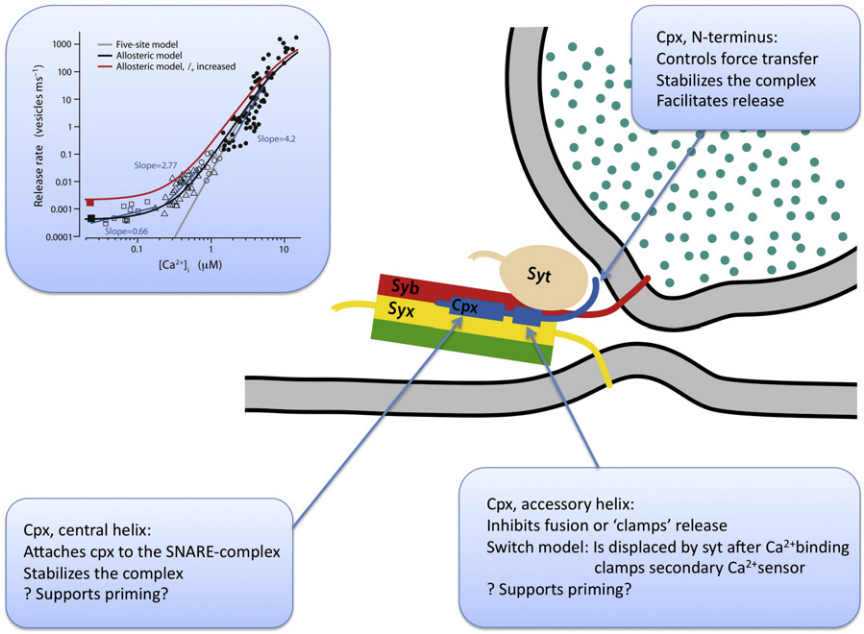

Figure 1. Schematic Diagram of the SNARE Complex and Complexin

Text panels refer to the three domains of Cpx (blue) and list the main functions attributed to them. A hyptothetical priming function is included for the accessory helix. This might be the case if this helix would not compete with Syt after priming but, would prevent the formation of nonproductive SNARE complexes, as observed in vitro (Fasshauer and Margittai, 2004).

The upper left panel shows caged-Ca^{2+} data (from Lou et al., 2005) with fits of an allosteric model (black) and a simple five-site model of release (gray). The red trace is the prediction of the allosteric model with a slightly increased energy of the resting state. This curve reproduced the data obtained in the presence of phorbol esters. Likewise, loss of Cpx or incorporation of an alternate Syt (or Doc2 protein) might shift the energy level, which would explain both increased spontaneous and asynchronous release.

that double-KD of the Cpx I and Cpx II isoforms increases spontaneous release. They further showed that this increase is Ca^{2+} dependent and that KD inhibits evoked release. The first two findings are in line with the switch model, but they require a modification to explain the Ca^{2+} dependence. Consequently, the authors postulate that Cpx clamps spontaneous release by blocking a so far unidentified secondary Ca^{2+} sensor for such release. The inhibition of evoked release by the KD is traced back to a reduction in priming, as probed by hypertonic (sucrose) stimulation. Furthermore, it is shown that effects of Syt1 KO and Cpx KD are additive. The authors conclude that the action of the two types of molecules on the secondary Ca^{2+} sensor is additive—while they are antagonistic in triggering (or preventing) fast release.

To further study the clamping action of Cpx, two mutations in the accessory helix region were designed to either increase the interaction with the SNARE complex (the so-called "superclamp" mutation) or to decrease it ("poorclamp" mutation). In vitro binding assays confirmed corresponding small changes in affinity to the SNARE comeplex. Rescue of KD revealed a deficit of the poorclamp mutant and a slightly enhanced clamping action for the super-clamp mutant, as expected. For other properties tested (evoked release and priming), the mutants behaved like wild-type Cpx. Two more mutations of well-conserved residues at the border between the accessory helix and the N terminus behaved quite normally, except that one of them (the so-called WW-mutation) did not clamp the spontaneous release. The two mutants that were poor clamps also displayed an increased relative contribution of delayed release, when NMDA responses were tested with short trains of stimuli. Performing such experiments at different extracellular [Ca^{2+}] showed that poor clamp was associated with an increased apparent Ca^{2+} affinity.

Finally, Yang et al. (2010) addressed the interaction between Cpx, Syt, and Syb. Maximov et al. (2009) had shown that a mutation in the linker region of Syb, connecting the SNARE motif with the membrane anchor, produced a phenotype similar to that of knockdown of Cpx, specifically increasing spontaneous release. With the modification of the Cpx-Syt-switch model (introducing the clamping of a secondary Ca^{2+} sensor to explain Ca^{2+} dependence of nonsynchronous release), the question arose of whether the increased spontaneous release of the Syb mutation also shows similar Ca^{2+} dependence. This was indeed found, leading to the conclusion that "deletions of both synaptotagmin and complexin, and mutation of the linker sequence … all disinhibit a secondary Ca^{2+} sensor."

Thus, an elaborate picture has evolved in which Cpxs and Syts partially complement each other but partially compete in the regulation of the SNARE complex (Figure 1). The experiments reported definitely support this interpretation. However, the underlying concept is quite complex and still does not offer straightforward explanations for some findings, such as the profound differences between KO and KD of Cpx and the multitude of differences in different model systems (Brose, 2008). Furthermore, no molecular mechanism for the clamping of the secondary sensor is yet emerging. Therefore, the question can be posed as to whether another perspective might offer a more unified and possibly simpler concept.

The alternative view of Cpx function, I would like to suggest, starts with some mechanistic considerations. It recognizes that the Ca^{2+} sensitivity of neurotransmitter release is remarkable. At the Calyx of Held, the rate of vesicle fusion changes by 6 to 7 orders of magnitude when [Ca^{2+}] changes from a basal level of about 50 nM to tens of μM (Lou et al., 2005). High rates of vesicle fusion are required to provide sufficient neurotransmitter release and precision of timing during the submillisecond episodes of Ca^{2+} inflow during action potentials. From a physical point of view, the real problem is to build a release machinery that is capable of generating such high release rates and at the same time to avoid intolerable release during periods of rest. Therefore, a primary goal in evolution must have been to develop

a release apparatus with high dynamic range. Given this requirement, it does not seem advisable to postulate on the one hand a clamp (as part of achieving the high dynamic range) and on the other hand an extra mechanism and Ca^{2+} sensor to produce asynchronous and spontaneous release that shortcircuits the clamp.

The second consideration is that the release apparatus actually does not need special mechanisms for generating the kinetic features of asynchronous release. The Ca^{2+} signal alone, being caused by localized Ca^{2+} influx, has sufficient complexity to generate the commonly observed sequence of synchronous release, followed by several kinetic components of asynchronous release. In particular, in the presence of mobile Ca^{2+} buffers, the decay of Ca^{2+} nanodomains displays two kinetic regimes: a first one in the submillisecond range, which is governed by diffusion of free Ca^{2+} and its equilibration with endogenous Ca^{2+} buffers, and a second one, buffered diffusion and Ca^{2+} sequestration, which depends on a multitude of factors, including fixed buffers, mobile buffers, pumps, and synapse geometry (reviewed in Neher, 1998).

How could a most simple release apparatus be built to provide a high dynamic range of release rates within a narrow [Ca^{2+}] range? Overwhelming evidence indicates that a small number of SNARE complexes constitute the core of such a release apparatus (Sudhof and Rothman, 2009). The "zippering-up" of the four-helix bundles will pull the membranes together until opposing forces acting on the membrane anchors of Syb and Syx prevent further zippering. At this point, Syt might be needed to support the zippering process. In the simplest case, Cpx may serve as an adaptor for this association (disregarding some in vitro evidence for competition between Cpx and Syt in SNARE binding). For our hypothetical simplest release apparatus, the energetics of this protein complex would have to be tuned such that the energy barrier for membrane fusion at low [Ca^{2+}] would be high enough to prevent intolerable resting release. Binding of Ca^{2+} to Syt would reduce the energy barrier by about 15 kcal/Mole (for a 10^{-6}- to 10^{7}-fold change in release rate) either by allosterically coupling the binding energy to the zippers or by rendering the membrane more fusogenic. If two to three Syt molecules, each binding two Ca^{2+} ions, contributed to this energy change, an allosteric model simulating this process would readily provide a "dose-response-curve" of release rate versus [Ca^{2+}], as measured by Lou et al. (2005) and Sun et al. (2007) (see Figure 1, upper left, for an example). Such a mechanism is analogous to many other examples of allosteric control, such as the gating of Ca^{2+}-activated K^{+} channels or the classical oxygen binding to hemoglobin.

Would such a simple model conform with the complex action of Cpx and the wealth of data on Syt/Cpx interactions? It would probably do so if one considered the release apparatus as an entity with components for each of which several isoforms are available. These are expressed in different cell types at different relative levels. Each cell type will express a set of isoforms that fit together and serve the specific needs of that particular cell type. For the Calyx of Held and other fast synapses, the set of subunits would be designed for high speed, large dynamic range, and relatively low affinity. At the *Drosphila* NMJ, reduction of spontaneous release may be priority; thus inhibitory functions via a Cpx clamp may be more prevalent. If one type of a component, which normally represents the major isoform, is knocked out, SNARE complex assembly may be reduced and slower, but eventually a competing isoform will take the place of the deleted one and form a release apparatus with energies of interaction different from those of the wild-type composition (Sørensen, 2009). Given that the wild-type composition has evolved to fulfill specialized tasks, it is highly likely that the altered composition will have properties that are more mainstream. For Syt-2-KO in a fast synapse, one would expect the dynamic range of the dose-response curve to be reduced, as observed (but interpreted differently) by Sun et al. (2007). This can be caused by a slightly increased energy barrier of the activated state (when Ca^{2+}-bound), or else by a decrease in the resting state. The latter would result in an increased rate of spontaneous release as observed with many knockouts. Likewise, point mutations would influence energy levels in a way that is not readily predicted (Jackson, 1987) but would most likely result in a decreased dynamic range (see Sørensen [2009], as well as Stein and Jahn [2009] for a discussion of the energetics of Syt-Cpx-SNARE interactions). It should also be pointed out that in the framework of an allosteric model an altered "apparent Ca^{2+} affinity" need not reflect changes at Ca^{2+}-binding sites. Any change in the energy levels may appear to include a change in affinity.

Returning to Cpx, the presumed role of a simple adaptor can well explain the fact that similar mutations (or KOs) in different preparations have quite different effects. It will depend on which isoforms of both Cpx and its binding partners are expressed in a given cell type, which might substitute for the knocked-out one, and how well that isoform does its job as an adaptor. In extreme cases (e.g., the present double knockdown or in triple knockouts of Cpx), some functionality of the release apparatus may remain in the absence of an adaptor. In any case, the energy levels of the resulting mix are likely to be different from those of the WT combination. Thus, instead of postulating separate and autonomous "clamp" and "triggering" functions to describe changes caused by mutations and substitutions, this view invokes changes in the energy levels of Ca^{2+}-bound and free states. The resulting release in the submicromolar Ca^{2+} concentration range would always be expected to be Ca dependent, since an allosteric mechanism implies a sigmoid dose-response curve with moderate Ca^{2+} sensitivity at both low and high Ca^{2+} concentrations and a steep transition in between (Figure 1). Thus, also from this point of view there is no need for a dedicated secondary sensor. Even if there were such additional Ca^{2+}-dependent interactions, which in the absence of a fast sensor would produce release similar to the asynchronous one, it would not be expected that they do the same in its presence. The KO would most likely have properties different from the WT in both the high and the low [Ca^{2+}] range, since it is the sum of all interactions that determines the energetics and kinetic properties of an allosteric machine.

Calling Cpx an adaptor is, of course, somewhat simplistic (Stein and Jahn, 2009). The data of Xue et al. (2007, 2010) and of Maximov et al. (2009) indicate

that separate domains of the molecule convey distinct, partially compensating energy contributions. They also imply that the N terminus is important for stability and energy transfer. In all cases, though, differences in interaction energies postulated in order to explain the experimental perturbations are minor. Typically, reaction rates in the low Ca^{2+} concentration regime are changed by factors of 2 to 5, corresponding to not more than 1.6 kT in net energy change. Assuming that there are two Cpx molecules involved in a release apparatus, the contribution of each would be just 0.8 kT—only a fraction of the energy of a hydrogen bond.

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Unloading Intracellular Calcium Stores Reveals Regionally Specific Functions

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Neuronal excitability can be modulated by release of intracellular calcium but the impact of calcium store depletion on intrinsic neuronal properties is unknown. In this issue of *Neuron*, Narayanan et al. describe an intrinsic plasticity that is depletion induced, is regionally restricted, and may protect neurons from pathological alterations in calcium signaling.

The view that intracellular calcium stores are passive reservoirs is a thing of the past. In the past few years, with the discovery of the molecular components responsible for refilling the stores, including the TRP family of channels (Ambudkar et al., 2007) and the Stim/Orai complex (Cahalan, 2009), the depletion of the calcium store in the endoplasmic reticulum (ER) has taken on new significance. As more members of the calcium signaling toolkit have been identified, additional functions have been assigned to specific cellular pathways (Choe and Ehrlich, 2006; Mikoshiba, 2007; Rizzuto and Pozzan, 2006). Along with the functional assignments is the understanding that modulation of the physiological signaling underlies these processes and, when disrupted, leads to pathological situations and chronic diseases.

Much of our knowledge of ER calcium storage and depletion has been obtained from studies using nonexcitable cells. Although neurons have been more difficult to study, in this issue of *Neuron*, Narayanan et al. (2010) elegantly examine the aftermath of ER calcium store depletion on hippocampal neurons. They found that ER calcium store depletion in CA1 pyramidal neurons of the hippocampus leads to an increase in functional *h* channels in the plasma membrane. The enhanced *h* current depended upon calcium release through inositol 1,4,5 trisphosphate receptors ($InsP_3Rs$), calcium entry through store operated channels (SOCs), and activation of protein kinase A (PKA). Increased *h* channel activity resulted in a persistent, perisomatic reduction in intrinsic neuronal excitability, which was accompanied by an increase in the optimal response frequency of the neuron. The authors suggest that this form of depletion-induced intrinsic plasticity could have a neuroprotective role in situations of pathological alterations of calcium signaling.

Remarkably, despite the global inhibition of the sarcoplasmic/endoplasmic reticular calcium ATP-ase (SERCA), changes in the electrical responses of the neurons were predominantly confined to the soma. What is the basis for the regional difference observed in the response to calcium store depletion? The authors suggest that this can be