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Comparative analysis of *Drosophila* and mammalian complexins as fusion clamps and facilitators of neurotransmitter release

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ABSTRACT

The SNARE-binding protein complexin (Cpx) has been demonstrated to regulate synaptic vesicle fusion. Previous studies are consistent with Cpx functioning either as a synaptic vesicle fusion clamp to prevent premature exocytosis, or as a facilitator to directly stimulate release. Here we examined conserved roles of invertebrate and mammalian Cpx isoforms in the regulation of neurotransmitter release using the *Drosophila* neuromuscular junction as a model synapse. We find that SNARE binding by Cpx is required for its role as a fusion clamp. All four mammalian Cpx proteins (mCpx), which have been demonstrated to facilitate release, also function as fusion clamps when expressed in *Drosophila cpx* null mutants, though their clamping abilities vary between isoforms. Moreover, expression of mCpx I, II or III isoforms dramatically enhance evoked release compared to mCpx IV or *Drosophila* Cpx. Differences in the clamping and facilitating properties of complexin isoforms can be partially attributed to differences in the C-terminal membrane tethering domain. Our findings indicate that the function of complexins as fusion clamps and facilitators of fusion are conserved across evolution, and that these roles are genetically separable within an isoform and across different isoforms.

Introduction

Neurotransmitter release is a tightly regulated process accompanied by cycles of assembly and disassembly of SNARE complexes during rounds of synaptic vesicle fusion (Littleton et al., 1998; Sollner et al., 1993). The requirement for the core SNARE machinery to form a parallel four-helix bundle to bring fusing membranes together is shared by most intracellular fusion processes. At synapses, SNARE-mediated fusion is uniquely regulated to allow rapid and calcium-triggered synaptic vesicle fusion. These adaptations to the core fusion machinery require synapse-specific SNARE-binding proteins, including Synaptotagmin 1 (Syt 1) and complexin (Cpx). Syt 1 is a synaptic vesicle protein that binds to SNARE complexes and membrane phospholipids in a calcium-dependent manner, functioning as the calcium sensor for fast synchronous neurotransmitter release (Geppert et al., 1994; Xu et al., 2007; Yoshihara and Littleton, 2002). In contrast with the established function for Syt 1, Cpx's role in synaptic vesicle fusion is less clear. Cpx is a small cytosolic α-helical protein that binds assembled SNARE complexes (Bracher et al., 2002; Chen et al., 2002; McMahon et al., 1995; Pabst et al., 2000). Association with SNAREs has been suggested to allow Cpx to function as a facilitator for synaptic vesicle fusion (Reim et al., 2001; Xue et al., 2007, 2008) or as a fusion clamp to prevent premature exocytosis in the absence of calcium (Huntwork and Littleton, 2007; Maximov et al., 2009).

Evidence supporting a role for Cpx as a synaptic vesicle fusion clamp has been suggested by both biochemical studies (Giraudo et al., 2006; Schaub et al., 2006) and genetic knockouts in *Drosophila* (Huntwork and Littleton, 2007). *Drosophila* has a single Cpx homolog (DmCpx) that is enriched in presynaptic nerve terminals. Deletion of *Drosophila cpx* results in a dramatic increase in spontaneous synaptic vesicle fusion, with a corresponding reduction in evoked release (Huntwork and Littleton, 2007), indicating that Cpx functions as a vesicle clamp to prevent calcium-independent fusion. The fusion clamp model is also supported by biochemical studies demonstrating that Cpx can inhibit SNARE-mediated fusion in cell–cell or liposome fusion assays (Giraudo et al., 2006, 2009; Schaub et al., 2006). In contrast to the single Cpx isoform in *Drosophila*, mammals have four Cpx genes encoded in their genome (Reim et al., 2005). Removal of Cpxs at mammalian synapses has been shown to increase spontaneous release in cortical neurons (Maximov et al., 2009) or decrease spontaneous release at autapses or GABAergic synapses (Reim et al., 2001; Strenzke et al., 2009; Xue et al., 2007, 2008). Similar to

Abbreviations: NMJ, neuromuscular junction; EJP, excitatory junctional potential; Cpx, complexin; m, mammalian; SNARE, soluble N-ethylmaleimide-sensitive fusion attachment protein receptor; Dm, *Drosophila melanogaster*; SEM, standard error of the mean; WT, wildtype; HRP, horse radish peroxidase.

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Drosophila, mammalian synapses lacking Cpxs also exhibit reduced evoked release. The distinct effects of Cpx on spontaneous release at different synapses within and across species suggest the protein may play multiple roles in synaptic vesicle trafficking depending upon the synaptic environment. Alternatively, Cpx may have a conserved role in fusion that manifests differently depending upon the presence or absence of other synaptic components that modify the fusion properties of synaptic vesicles. A key test of these two models is determining whether the distinct roles of Cpx in clamping fusion and promoting release can be genetically separated, which would argue for multiple roles of Cpx during the synaptic vesicle cycle. Recent studies suggest Cpx contains both facilitating and inhibitory functions that are differentially manifest between invertebrate and mammalian isoforms (Xue et al., 2009).

To further examine these alternative models for Cpx function, we extended our previous analysis of *Drosophila* Cpx and tested whether mammalian Cpx (mCpx) isoforms can functionally substitute for the loss of Cpx at *Drosophila* neuromuscular junction (NMJ) synapses. We demonstrate that SNARE binding by Cpx is required for its roles as both a fusion clamp and a facilitator of vesicle fusion. All mCpx isoforms can partially rescue the *cpx* mini frequency phenotype, suggesting that the ability of Cpx to act as a fusion clamp is evolutionarily conserved. Strikingly, mCpx IV, like the *Drosophila* homolog, can potently clamp spontaneous release compared to the other mCpx isoforms. To examine properties that might confer the ability of mCpx IV and DmCpx to function as potent fusion clamps, we investigated the C-terminal CAAX-box prenylation motif conserved in mCpx III, mCpx IV, and DmCpx. We find that the CAAX-box motif is necessary but not sufficient for Cpx to act as a fusion clamp. Finally, we show that mCpxs I, II, and III dramatically promote enhanced evoked release, whereas mCpx IV or DmCpx do not. Unexpectedly, expression of DmCpx lacking the CAAX-box motif in the *cpx* nulls enhances evoked release compared to WT DmCpx, suggesting that prenylation may partially mask facilitatory properties of Cpx. These experiments suggest that the dual function of Cpxs as fusion clamps and facilitators of release is evolutionarily conserved, requires SNARE binding, and can be genetically separated.

Results

DmCpx function as a fusion clamp requires SNARE binding

Genetic approaches have suggested two potential roles for Cpxs in synaptic exocytosis — as a fusion clamp and/or a facilitator of vesicle fusion. To further define the role of Cpx in neurotransmitter release, we examined similarities and differences in the function of *Drosophila* and mammalian Cpx isoforms at *Drosophila* NMJ synapses. Cpx is a small cytoplasmic protein with a central α-helix that binds the SNARE complex (Bracher et al., 2002; Chen et al., 2002) (Fig. 1A). The SNARE-binding domain of Cpx is highly conserved across evolution (Brose, 2008) and is essential for Cpx's role in facilitating synaptic vesicle fusion at mammalian synapses. Lysine 75 and tyrosine 76 flank the SNARE-binding domain and are critical for Cpx–SNARE binding (Bracher et al., 2002; Chen et al., 2002; Xue et al., 2007, 2009). To determine if DmCpx must also engage SNARE complexes for its role in clamping spontaneous synaptic vesicle fusion, we generated transgenic lines expressing DmCpx with lysine 75 and tyrosine 76 mutated to alanine ($DmCpx^{K75A,Y76A}$) in the *cpx* null background under control of the GAL4–UAS system. We used the phiC31–attP recombination

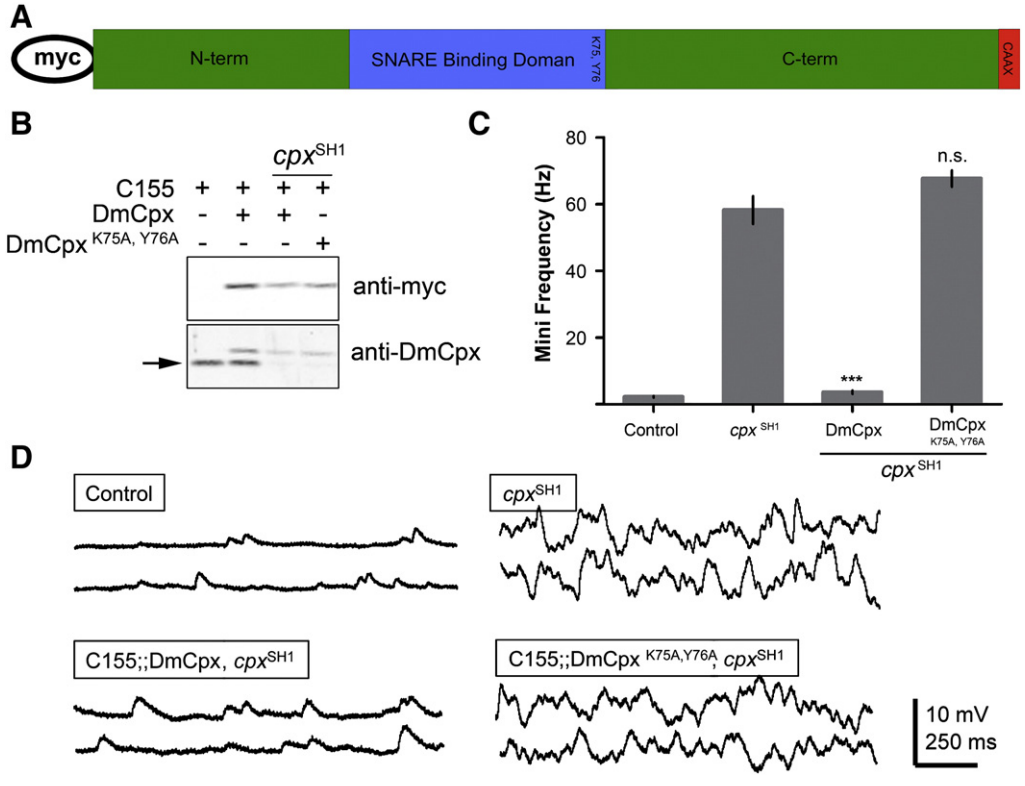

Fig. 1. DmCpx function as a fusion clamp requires the SNARE-binding residues lysine 75 and tyrosine 76. (A) Schematic of Cpx protein domains. All transgenic lines generated in this study encoded a Cpx with an N-terminal myc tag. The SNARE-binding domain is indicated in blue, and the localization of K75 and Y76 is shown. The C-terminal CAAX-box motif contained in some Cpx isoforms is shown in red. (B) The $DmCpx^{K75A,Y76A}$ transgenic protein is expressed at levels similar to the WT DmCpx transgenic protein and endogenous Cpx in lysates from adult transgenic heads. The top blot shows western probed with anti-myc antisera that recognizes the transgenic proteins driven by $C155^{elav\text{-}Gal4}$ in WT or cpx^{SH1} background as indicated. The bottom blot is probed with anti-*Drosophila* Cpx antisera, which recognizes both endogenous and transgenic protein. Myc-tagged Cpx runs slower and can be separated from endogenous Cpx (indicated by the arrow). (C) Summary of mean mini frequency (Hz ± SEM). n = control (10), cpx^{SH1} (7), DmCpx (8), $DmCpx^{K75A,Y76A}$ (6). Statistical significance was determined by Student's t-test. (D) Sample traces of minis in control, cpx^{SH1}, and rescue lines expressing WT DmCpx or $DmCpx^{K75A,Y76A}$.