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# Molecular mechanisms of COMPLEXIN fusion clamp function in synaptic exocytosis revealed in a new *Drosophila* mutant

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ABSTRACT

The COMPLEXIN (CPX) proteins play a critical role in synaptic vesicle fusion and neurotransmitter release. Previous studies demonstrated that CPX functions in both activation of evoked neurotransmitter release and inhibition/clamping of spontaneous synaptic vesicle fusion. Here we report a new *cpx* mutant in *Drosophila melanogaster*, $cpx^{1257}$, revealing spatially defined and separable pools of CPX which make distinct contributions to the activation and clamping functions. In $cpx^{1257}$, lack of only the last C-terminal amino acid of CPX is predicted to disrupt prenylation and membrane targeting of CPX. Immunocytochemical analysis established localization of wild-type CPX to active zone (AZ) regions containing neurotransmitter release sites as well as broader presynaptic membrane compartments including synaptic vesicles. Parallel biochemical studies confirmed CPX membrane association and demonstrated robust binding interactions of CPX with all three SNAREs. This is in contrast to the $cpx^{1257}$ mutant, in which AZ localization of CPX persists but general membrane localization and, surprisingly, the bulk of CPX–SNARE protein interactions are abolished. Furthermore, electrophysiological analysis of neuromuscular synapses revealed interesting differences between $cpx^{1257}$ and a *cpx* null mutant. The *cpx* null exhibited a marked decrease in the EPSC amplitude, slowed EPSC rise and decay times and an increased mEPSC frequency with respect to wild-type. In contrast, $cpx^{1257}$ exhibited a wild-type EPSC with an increased mEPSC frequency and thus a selective failure to clamp spontaneous release. These results indicate that spatially distinct and separable interactions of CPX with presynaptic membranes and SNARE proteins mediate separable activation and clamping functions of CPX in neurotransmitter release.



## Introduction

It is widely accepted that SNARE proteins function at the core of the neurotransmitter release apparatus, where they promote exocytotic fusion of neurotransmitter-filled synaptic vesicles with the presynaptic plasma membrane (Jahn and Scheller, 2006). However, defining the mechanisms which provide precise and rapid regulation of synaptic vesicle fusion remains among the foremost problems in cellular and molecular neuroscience. The identification of CPX as a protein which binds and regulates SNARE complexes (Ishizuka et al., 1995; McMahon et al., 1995) has advanced our understanding of these mechanisms (Brose, 2008; Neher, 2010; Rizo and Rosenmund, 2008; Stein and Jahn, 2009; Südhof and Rothman, 2009). Notably, CPX can both promote SV fusion evoked by a presynaptic action potential and suppress or "clamp" spontaneous vesicle fusion. Recent models suggest that specific domains of CPX (Fig. 1A) contribute to different aspects of synaptic vesicle fusion (Hobson et al., 2011; Martin et al., 2011; Maximov et al., 2009; Reim et al., 2001; Strenzke et al., 2009; Tang et al., 2006; Xue et al., 2007, 2009, 2010; Yang et al., 2010). Whereas a "central helix" which binds SNARE complexes (Bracher et al., 2002; Chen et al., 2002) is absolutely required for CPX function, other domains appear to mediate specific aspects of CPX activity (Rizo and Rosenmund, 2008; Stein and Jahn, 2009). For example, recent studies have shown that the CPX C-terminus is specifically required for the clamping function (Buhl et al., 2013; Cho et al., 2010; Kaeser-Woo et al., 2012; Martin et al., 2011; Xue et al., 2009). Of particular relevance to the present study is a specific CaaX motif found at the extreme C-terminus of several mammalian and *Drosophila* CPX isoforms. This motif has been shown to mediate CPX prenylation [a form of lipid modification; (Omer and Gibbs, 1994; Resh, 2006)] and has been implicated in both targeting CPX to membranes (Reim et al., 2005) and the CPX clamping function (Cho et al., 2010; Xue et al., 2009). The form of prenylation demonstrated for mammalian CPX isoforms (CPX3 and 4) is farnesylation (Reim et al., 2005), consistent with previous studies indicating that one of several specific residues in the X position of the CaaX motif (A,C,M,Q,S) selectively mediates farnesylation (Omer and Gibbs, 1994).

*Abbreviations:* AZ, active zone; BRP, BRUCHPILOT; CPX, COMPLEXIN; DLM, dorsal longitudinal flight muscle; EPSC, excitatory postsynaptic current; mEPSC, miniature excitatory postsynaptic current; mEPSP, miniature excitatory postsynaptic potential; NSYB, neuronal-SYNAPTOBREVIN; SV, synaptic vesicle; SYT, SYNAPTOTAGMIN; SYX, SYNTAXIN.

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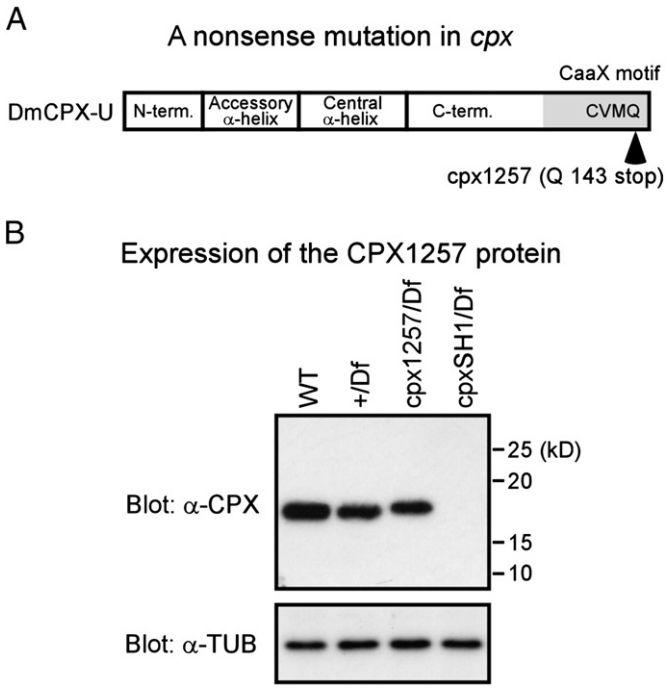


**Fig. 1.** A new *cpx* mutant, $cpx^{1257}$. (A) Schematic of *Drosophila* CPX (isoform DmCPX-U) and the nonsense mutation in $cpx^{1257}$. The $cpx^{1257}$ mutation removes the last C-terminal amino acid of CPX, corresponding to the X in the CaaX motif required for farnesylation. This mutation is isoform-specific in that it occurs in an alternative exon encoding the C-terminus of CPX (highlighted in gray; see also Fig. 2). The domain organization shown was adapted from a previous study (Xue et al., 2007). The accession number for DmCPX-U is AY121629. (B) Western analysis of $cpx^{1257}$. Western blot of fly head homogenates prepared from wild type (WT) and *cpx* hemizygotes heterozygous for *Df(3R)Exel6140* (*Df*) which removes the *cpx* locus, as well as $cpx^{1257}/Df$ and $cpx^{SH1}/Df$. Although $CPX^{1257}$ migrates at a slightly higher relative molecular mass in comparison to wild-type CPX, the levels in the $cpx^{1257}/Df$ and $+/Df$ samples are similar, indicating normal CPX expression in $cpx^{1257}$. CPX is absent in $cpx^{SH1}/Df$ hemizygotes. Tubulin (TUB) was used as an internal loading control.

This progress is extended by new insights gained from the present study, in which the isolation and characterization of a new *cpx* mutant further define the in vivo molecular basis of CPX functions and interactions within the neurotransmitter release apparatus. This study reveals a specific subcellular distribution for CPX within the presynaptic terminal and a role for C-terminal farnesylation in mediating both association of CPX with presynaptic membranes and CPX clamping of spontaneous synaptic vesicle fusion.

## Results

### *Genetic and molecular characterization of a new cpx mutant*

Further genetic analysis to examine the in vivo molecular mechanisms of CPX function was pursued through a forward genetic screen for new mutant alleles of the single *Drosophila cpx* gene. To complement a previously reported *cpx* null mutant (Huntwork and Littleton, 2007), this screen was intended to recover hypomorphic and conditional alleles that may further define the in vivo molecular determinants of CPX function. A screen was performed using chemical mutagenesis and subsequent screening for *cpx* mutants in F2 progeny carrying a mutagenized third chromosome *in trans* to a deficiency (deletion) which removes *cpx* (see Experimental methods). The screen consisted of examining motor behavior at the elevated temperature to detect hypomorphic or temperature-sensitive (TS) phenotypes. One new mutant, initially referred to *1257*, was recovered on the basis of its severe lack of motor co-ordination at 38 °C.

On the basis of genetic complementation testing with the *cpx* null mutant, *1257* was confirmed to be a new allele of *cpx* and named $cpx^{1257}$. Sequence analysis of the *cpx* ORF revealed the molecular lesion in $cpx^{1257}$. Remarkably, this mutation removes only the last C-terminal amino acid of CPX, which is a Q in the X position of the C-terminal CaaX sequence (Fig. 1A). This residue is required for farnesylation of certain mammalian CPX isoforms (CPX3 and 4) and has been implicated in their membrane targeting (Reim et al., 2005). Western analysis of the $cpx^{1257}$ mutant (Fig. 1B) demonstrated wild-type CPX protein levels in $cpx^{1257}$ [comparing the hemizygous conditions, $cpx^{1257}/Df(3R)Exel6140$ and $+/Df(3R)Exel6140$]. Thus the $cpx^{1257}$ phenotype appears to reflect the properties of the mutant protein rather than its expression level. Note that $CPX^{1257}$ migrates at a slightly higher relative molecular mass in comparison to wild-type CPX (Fig. 1B), most likely because of altered post-translational processing. Finally, the $cpx^{1257}$ mutation is isoform-specific in that it occurs in an alternative exon which is present in most isoforms expressed from the single *Drosophila cpx* gene [(Buhl et al., 2013) and see Fig. 2A and Experimental methods]. Incidentally, the altered migration of $CPX^{1257}$ in Western analysis also permits the conclusion that CPX isoforms containing this alternative exon, and thus those affected by the $cpx^{1257}$ mutation, are predominant in the nervous system (Fig. 2B). Lastly, final confirmation that $cpx^{1257}$ is an allele of

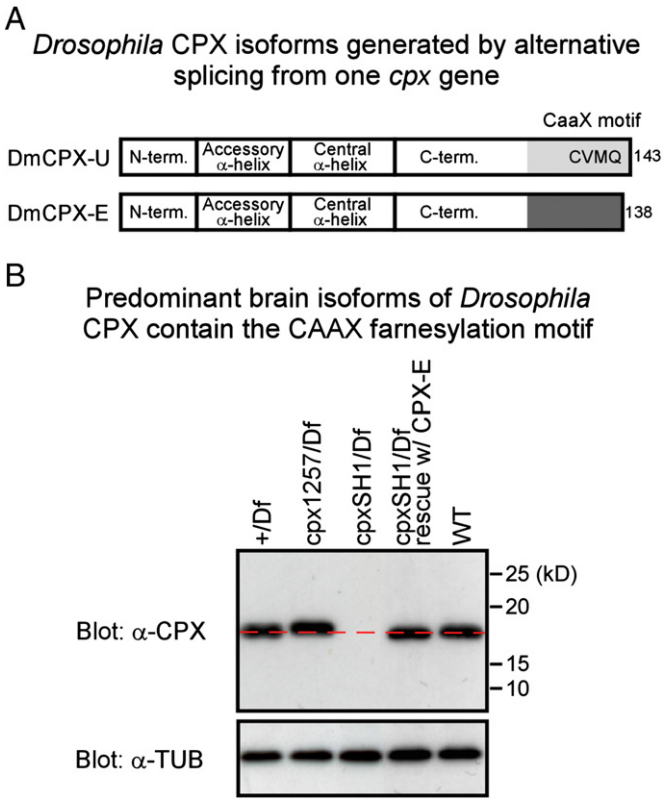


**Fig. 2.** CPX isoforms contain distinct C-terminal domains. (A) Two *Drosophila* CPX (DmCPX) isoforms generated by alternative splicing from the single *cpx* gene. A main difference between them is that the C-terminus (shown in different shades of gray) is encoded by two different alternative exons. DmCPX isoforms U and E, respectively, represent isoforms predicted to be farnesylated (CaaX containing) or not farnesylated (CaaX lacking). The exon containing the CaaX motif is present in most isoforms (http://www.flybase.org, see also the Experimental methods section — *Relevant information about cpx alternative exons and splice variants*). (B) Predominant brain isoforms of *Drosophila* CPX contain the CaaX farnesylation motif. Western analysis of fly head homogenates prepared from WT, *cpx* hemizygotes heterozygotes for *Df(3R)Exel6140* (+/Df), $cpx^{1257}/Df$, $cpx^{SH1}/Df$ and $cpx^{SH1}/Df$ rescued by neural expression of the wild-type CPX isoform E. Rescue of $cpx^{SH1}$ was carried out in *Appl-GAL4;;UAS-cpx* $cpx^{SH1}/Df(3R)Exel6140$ flies. The predominance of CaaX-containing CPX isoforms in the *Drosophila* brain is inferred from the following observations. First, CPX-E migrates at a slightly lower relative molecular mass in comparison to wild-type CPX from $+/Df$ and WT flies and does not appear to be detected in head homogenates containing endogenous CPX. Second, the altered migration of $CPX^{1257}$ appears to shift all of the detectable endogenous CPX signal. Tubulin (TUB) was used as an internal loading control.