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Differential regulation of evoked and spontaneous neurotransmitter release by C-terminal modifications of complexin

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ABSTRACT

Complexins are small α-helical proteins that modulate neurotransmitter release by binding to SNARE complexes during synaptic vesicle exocytosis. They have been found to function as fusion clamps to inhibit spontaneous synaptic vesicle fusion in the absence of Ca^{2+}, while also promoting evoked neurotransmitter release following an action potential. Complexins consist of an N-terminal domain and an accessory α-helix that regulates the activating and inhibitory properties of the protein, respectively, and a central α-helix that binds the SNARE complex and is essential for both functions. In addition, complexins contain a largely unstructured C-terminal domain whose role in synaptic vesicle cycling is poorly defined. Here, we demonstrate that the C-terminus of *Drosophila* complexin (DmCpx) regulates localization to synapses and that alternative splicing of the C-terminus can differentially regulate spontaneous and evoked neurotransmitter release. Characterization of the single *DmCpx* gene by mRNA analysis revealed expression of two alternatively expressed isoforms, DmCpx7A and DmCpx7B, which encode proteins with different C-termini that contain or lack a membrane tethering prenylation domain. The predominant isoform, DmCpx7A, is further modified by RNA editing within this C-terminal region. Functional analysis of the splice isoforms showed that both are similarly localized to synaptic boutons at larval neuromuscular junctions, but have differential effects on the regulation of evoked and spontaneous fusion. These data indicate that the C-terminus of *Drosophila* complexin regulates both spontaneous and evoked release through separate mechanisms and that alternative splicing generates isoforms with distinct effects on the two major modes of synaptic vesicle fusion at synapses.

Introduction

A conserved vesicle trafficking machinery made up of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and SM proteins drives membrane fusion in multiple cellular compartments (Südhof and Rothman, 2009). At synapses, the fusion of neurotransmitter containing vesicles with the plasma membrane is tightly regulated to allow for precise communication within the nervous system. Several SNARE-binding accessory proteins that modulate the activity of the core machinery have evolved to provide synapse-specific requirements for synaptic vesicle fusion. The best studied of these accessory proteins is the vesicular Ca^{2+} sensor synaptotagmin 1 (Syt 1), which binds to SNARE complexes and membrane phospholipids in a Ca^{2+}-dependent manner to allow for fast synchronous neurotransmitter release in response to Ca^{2+} (Geppert et al., 1994; Xu et al., 2007; Yoshihara and Littleton, 2002). In contrast to Syt 1, the precise function of complexin (Cpx) in synaptic vesicle fusion is still being elucidated. Cpxs are small, α-helical proteins identified based on their ability to bind the assembled SNARE complex with 1:1 stoichiometry (Bracher et al., 2002; Chen et al., 2002; McMahon et al., 1995; Pabst et al., 2000).

Numerous studies have suggested that Cpx acts both to inhibit spontaneous neurotransmitter release in the absence of Ca^{2+} (Hobson et al., 2011; Huntwork and Littleton, 2007; Martin et al., 2011; Maximov et al., 2009) and to promote evoked neurotransmitter release (Cai et al., 2008; Hobson et al., 2011; Huntwork and Littleton, 2007; Martin et al., 2011; Maximov et al., 2009; Reim et al., 2001; Xue et al., 2007, 2008). Data from biochemical studies (Giraudo et al., 2006; Schaub et al., 2006), genetic knock-out studies in *Drosophila* and *Caenorhabditis elegans* (Hobson et al., 2011; Huntwork and Littleton,

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

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2007; Martin et al., 2011) and genetic knock-down studies in mice (Maximov et al., 2009) have supported the role of Cpx as an inhibitor of spontaneous neurotransmitter release. Genetic deletion of the single Cpx homolog in *Drosophila* (DmCpx) results in a dramatic increase in the frequency of spontaneous vesicle fusion events (minis) at the larval neuromuscular junction (NMJ) (Cho et al., 2010; Huntwork and Littleton, 2007). Similarly, the frequency of tonic fusion events at the *C. elegans* NMJ is increased in genetic knock-outs of the primary Cpx homolog (CeCpx-1) (Hobson et al., 2011; Martin et al., 2011). Unlike flies and worms, mammals have four Cpx genes with distinct expression patterns in the nervous system (Reim et al., 2005). RNAi knock-down of Cpxs in mouse cortical cultures increases spontaneous neurotransmitter release (Maximov et al., 2009). However, genetic knock-out of Cpxs results in decreased spontaneous neurotransmitter release at hippocampal autapses and GABA-/glycinergic synapses, but not at striatal autapses (Strenzke et al., 2009; Xue et al., 2007, 2008).

In contrast to the different findings on spontaneous fusion, studies have consistently shown that Cpx is necessary to promote evoked Ca^{2+}-dependent neurotransmitter release. These data indicate that Cpx has distinct effects on different modes of neurotransmitter release and plays several roles during the multi-step process of synaptic vesicle fusion. Structure–function studies suggest that different domains of Cpx contribute to specific steps in synaptic vesicle trafficking. A central helix within Cpx is necessary for SNARE binding as determined by crystallography (Bracher et al., 2002; Chen et al., 2002). Cpx constructs that lack this domain or key binding residues within it are non-functional (Cho et al., 2010; Giraudo et al., 2008; Martin et al., 2011; Maximov et al., 2009; Xue et al., 2007). The N-terminus, meanwhile, appears to contain both facilitatory and inhibitory domains that may be differently used at mammalian and invertebrate synapses (Giraudo et al., 2009; Hobson et al., 2011; Martin et al., 2011; Xue et al., 2007, 2009, 2010). In contrast, the function of the C-terminus is poorly understood. Biochemical studies have shown that the C-terminus inhibits SNARE-mediated cell fusion but promotes cell-mediated liposome fusion (Giraudo et al., 2008; Malsam et al., 2009). In addition, Cpx constructs that lack the C-terminus are functional in hippocampal autapses, yet fail to rescue the increased tonic neurotransmitter release observed at the *C. elegans* NMJ in *cpx-1* null mutants, suggesting that the C-terminus may act to inhibit neurotransmitter release at some synapses. Recent studies of several mammalian Cpx isoforms suggest the C-terminal domain may differentially regulate clamping versus activation properties of different isoforms (Kaeser-Woo et al., 2012). Given these divergent results, additional characterization of the C-terminus is needed to define its precise role in synaptic transmission.

In this study, we analyzed the function of the C-terminus of DmCpx. Using a chemical mutagenesis approach, we isolated a *Cpx* allele with an early stop codon that truncates the far C-terminus. These mutants show reduced Cpx protein levels and mislocalize Cpx at synaptic boutons at *Drosophila* larval NMJs. We subsequently identified two alternatively spliced isoforms, DmCpx7A and DmCpx7B, which vary in the far C-terminus, with additional C-terminal variation created through RNA editing of DmCpx7A. Although DmCpx7A predominates at the mRNA level in larvae and adults, both isoforms are expressed in the developing nervous system and their mRNA expression is activity-regulated. In transgenic rescue experiments, we show that DmCpx7A and DmCpx7B are similarly localized to synaptic boutons at the larval NMJ. However, we find that DmCpx7A and DmCpx7B have different effects on spontaneous and evoked neurotransmitter release, with DmCpx7A being a better inhibitor of spontaneous release and DmCpx7B functioning as a better facilitator of evoked release. DmCpx7A contains a C-terminal membrane tethering prenylation domain, while DmCpx7B does not. We propose a model in which C-terminal modification regulates the effects of Cpx on different modes of neurotransmitter release.

Results

The C-terminus of DmCpx is necessary for protein stability, localization and function

We performed an ethane methylsulfonate (EMS) non-complementation mutagenesis screen with the cpx^{SH1} null mutant to isolate additional *Cpx* alleles in *Drosophila*. cpx^{572} was identified in a screen of 5000 mutagenized lines as a loss-of-function mutation. Sequence analysis revealed that the cpx^{572} allele contains a small deletion near the end of exon 6, leading to a premature stop codon and deletion of the final ~25 residues of the Cpx protein (Fig. 1A). Similar to the cpx^{SH1} null allele (Huntwork and Littleton, 2007), cpx^{572} is semi-lethal. Homozygous adult cpx^{572} escapers are uncoordinated and ataxic, though they are less severely affected than null animals. Western blot analysis indicates that a truncated form of Cpx protein is present in head extracts from cpx^{572} adults. The truncated Cpx protein is expressed at <20% of wild type levels (Fig. 1B), suggesting that the far C-terminus regulates Cpx protein stability in vivo.

We next evaluated Cpx protein localization and function at larval NMJs of cpx^{572} mutants. Although weak Cpx expression could be detected within motor axons, Cpx failed to accumulate in synaptic boutons at similar levels to the wildtype protein when examined by confocal microscopy (Fig. 1C). Imaging at higher laser power revealed that the remaining Cpx found at mutant cpx^{572} NMJs was distributed in puncta, rather than being localized diffusely in the bouton as observed in controls (Fig. 1D). The Cpx puncta found at cpx^{572} NMJs resided near, but did not completely overlap active zones, as revealed by co-staining for the active zone protein Bruchpilot. The residual Cpx did not overlap with peri-active zone proteins such as Fas2 (Fig. 1D). These data indicate that the far C-terminus of Cpx regulates its subsynaptic localization. In addition to mislocalization of Cpx at synapses, cpx^{572} larvae displayed disruptions in synaptic transmission at the NMJ, with a slightly less severe phenotype than observed in cpx^{SH1} null larvae (Fig. 2). cpx^{572} mutants show a large elevation in the frequency of spontaneous release (Fig. 2A, B), and a milder reduction in the amplitude of the evoked response compared to nulls (Fig. 2C, D). Compared to cpx^{SH1} null mutants, cpx^{572} mutants also showed milder defects in synaptic depression during short (Fig. 2E, F) and long (Fig. 2G–I) stimulation trains. These data indicate that the C-terminus regulates both subsynaptic localization and function of Cpx, and that the cpx^{572} mutant is a hypomorphic allele.

The Drosophila Cpx locus generates multiple splice isoforms

To further investigate the function of the C-terminus of DmCpx, we used cDNA analysis to detect any endogenous sequence variation present at the *Cpx* genomic locus. Unlike mammals, which have four Cpx genes, there is only a single *Cpx* gene in *Drosophila*. An analysis of the genomic sequence compared to sequenced expressed sequence tag (EST) clones revealed multiple alternative splicing predictions for *DmCpx* (Fig. 3A). The genomic locus is predicted to encode 12 different transcripts produced by alternative splicing of the first exon, which contains a large portion of the 5′ untranslated region (UTR) of the *cpx* mRNA, but no coding sequence. Alternative splicing of the first exon is well supported by sequences of ESTs that correspond to those found in the alternate first exons. In general, the 5′ UTR of mRNA sequences can regulate both translation initiation and mRNA stability (Pickering and Willis, 2005). The wide variety of 5′ UTRs in *cpx* transcripts suggests that the timing, level, and/or location of Cpx expression is likely to be tightly regulated in *Drosophila*. We also identified alternative splicing that altered coding exons within Cpx. Notably, the far C-terminus is encoded by one of two alternatives for exon 7, which we term exon 7A and exon 7B. We PCR amplified and sequenced individual cDNAs from the ATG start site in exon 3 to the stop codon in exon 7A or exon 7B. Although alternative splicing