

Evolutionary conservation of complexins: from choanoflagellates to mice

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Abstract

Complexins are synaptic SNARE complex-binding proteins that cooperate with synaptotagmins in activating Ca²⁺-stimulated, synaptotagmin-dependent synaptic vesicle exocytosis and in clamping spontaneous, synaptotagmin-independent synaptic vesicle exocytosis. Here, we show that complexin sequences are conserved in some non-metazoan unicellular organisms and in all metazoans, suggesting that complexins are a universal feature of metazoans that predate metazoan evolution. We show that complexin from *Nematostella vectensis*, a cnidarian sea anemone far separated from mammals in metazoan evolution, functionally replaces mouse complexins in activating Ca²⁺-triggered exocytosis, but is unable to clamp spontaneous exocytosis. Thus, the activating function of complexins is likely conserved throughout metazoan evolution.

Keywords evolution; membrane fusion; SNARE proteins; synapse; synaptotagmin

Subject Categories Evolution; Membrane & Intracellular Transport

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Introduction

Neurotransmitter release is mediated by synaptic vesicle fusion that is triggered by Ca²⁺ binding to synaptotagmins. SNARE and SM proteins catalyze fusion by forming a tight complex that forces the membranes into close proximity. Synaptotagmins promote fusion via a Ca²⁺-dependent interaction with both phospholipid membranes and SNARE/SM protein complexes [1]. However, synaptotagmins do not act alone but require complexins as cofactors. Complexins are small (~130 residues) SNARE-binding proteins. In vertebrates, complexins primarily perform an activating function in release by rendering the SNARE complex competent for Ca²⁺ triggering of fusion and boosting the priming of synaptic vesicles. In

addition, vertebrate complexins clamp spontaneous release in most synapses. In invertebrates, however, complexins appear to be more prominently involved in clamping release, although an activating function has also been observed [2].

These findings led to the hypothesis that evolutionarily, complexins may have originated as a clamp in invertebrates and subsequently acquired activator functions in vertebrates [3]. Other studies, however, suggested that invertebrate and vertebrate complexins similarly act as both clamps and activators in release [4–8], and *in vitro* reconstitutions with mammalian complexins reproduce both activities [9–12]. Moreover, no complexins from organisms that are evolutionarily more ancient than *D. melanogaster* and *C. elegans* have been examined.

Structurally, complexins contain an N-terminal unstructured region followed by an accessory α -helix, a SNARE-binding central α -helix, and a longer unstructured C-terminal region. The three functions of vertebrate complexins (priming, Ca²⁺ triggering, and clamping) exhibit distinct sequence requirements, such that the N-terminal region is selectively necessary for Ca²⁺ triggering of exocytosis, the accessory α -helix for clamping, and the C-terminal unstructured region is involved in both clamping and priming but not Ca²⁺ triggering, while the central α -helix is required for all complexin functions [5,8,13–15]. The differential sequence dependence of complexin functions strongly suggests that these functions are mechanistically distinct.

Here, we show that complexin sequences are not only encoded by all metazoan genomes, but are also present in the genomes of a subset of unicellular organisms that are evolutionarily older than metazoans, such as choanoflagellates. We found that the genomes of primitive metazoans, such as that of the sea anemone *Nematostella vectensis*, encode one or two complexin genes. *Nematostella* is a cnidarian that belongs to the simplest eumetazoans and develops primitive neuron-like cells, but lacks a central nervous system [16]. Furthermore, we demonstrate that re-introduction of *Nematostella* complexin-1 into complexin-deficient mouse neurons fully rescued the inactivation of evoked neurotransmitter release, but did not reverse the unclamping of spontaneous mini release. Thus, *Nematostella* complexin-1—similar to mouse complexin-1—functions as an

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activator of release, but may lack clamping activity. Moreover, we demonstrate that *Nematostella* complexin-1 exhibits a similar structure/function dependence as vertebrate complexins. Our data suggest that complexins activate exocytosis by mechanisms that are conserved throughout metazoan evolution and likely originate in evolution prior to the emergence of metazoans.

Results and Discussion

Complexin evolution

We performed sequence similarity searches for complexins by PSI-BLAST. Complexin sequences were found in all major groups of metazoans (four basal metazoan groups: Porifera, Ctenophora, Cnidaria, and Placozoa and three Bilateria groups: Ecdysozoa, Lophotrochozoa, and Deuterostomia). Vertebrate organisms have four or more complexin sequences, possibly contributed by whole genome duplications. Invertebrate organisms, on the other hand, often have only one complexin gene. A few non-vertebrate metazoans have two complexins, such as *Caenorhabditis elegans*, sea urchin (*Strongylocentrotus purpuratus*), and *Nematostella vectensis*, which may be attributed to lineage-specific gene duplication events (Fig 1).

Complexin sequences encode four domains: a short N-terminal domain with a conserved pattern of hydrophobic and positively charged residues, a charged accessory α -helix, a central α -helix that binds to SNARE complexes, and a C-terminal region that often accounts for most of the complexin sequence (Fig 1A). The N-terminal domain and central α -helix exhibit the most conservation across species, whereas the C-terminal region is the most variable (Fig 1B). Negatively charged residue patches are often present in the accessory α -helix (all complexins) and the C-terminal domain (mostly complexins from vertebrates). Many but not all complexin sequences have a C-terminal CAAX motif and are likely isoprenylated. All four domains were detected in all complexin sequences (except zebrafish Complexin 4b that lacks the N-terminal domain), suggesting that they are obligatory complexin components (Fig 1B).

Surprisingly, we also observed that a few non-metazoan single-cell organisms belonging to groups such as Choanoflagellata, Filasterea, and Nuclearia, but not in Fungi, Amoebozoa, and Unikonta, contain single complexin sequences with an apparently similar domain organization (Fig 1). Specifically, we detected complexin sequences in the choanoflagellate *Monosiga brevicollis*, the cellular slime mold *Fonticula alba*, and the filasterean *Capsaspora owczarzewski*, as previously noted in the Supplementary Materials of

Burckhard *et al* [17]. The unicellular complexins include all of the typical complexin features, including the N-terminal sequence containing the typical amphipathic pattern of hydrophobic and positively charged residues, the classical central α -helix that is highly similar to that of other complexins, and a C-terminal region with a canonical isoprenylation sequence (CAAX box). The major difference between the non-metazoan and metazoan complexins is that the unstructured C-terminal half of the molecule that is involved in clamping and priming in mammalian complexins [5,8,18] is shorter and more variable. However, this part is the least conserved sequence in all complexins and probably exerts a primarily modulatory function [18].

We next sought to investigate the relation of the various complexin sequences we identified by constructing a phylogenetic tree. The small size and limited positions in complexins coupled with sequence divergence make phylogenetic signals weak, and the phylogenetic construction by MOLPHY did not yield significant supports in most groups (Fig 2). Nevertheless, the analyses clearly show that there is no clear-cut evolutionary division of complexins into evolutionarily distinct classes. The most robust separation of complexins into classes was observed for the vertebrate sequences which formed two major groups, one including mouse complexin-1 and -2 (without a CAAX motif), and the other including mouse complexin-3 and -4 (with a CAAX motif).

Nematostella complexin-1 is a functional activator of neurotransmitter release in mouse neurons

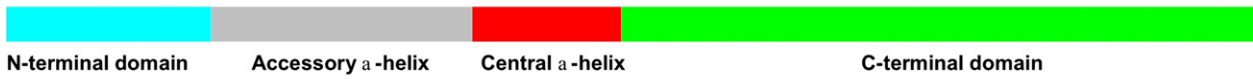
Given the small size of complexins, their surprising conservation in the most primitive metazoans and even in some unicellular organisms raises the question whether the complexins observed in these species actually function as complexins. To address this question, we focused on two complexin sequences that we had identified in the *Nematostella vectensis* genome [19] (Figs 1 and EV1). One of the two *Nematostella* complexins (complexin-1 [referred to as “nvCpx1”]) contains a C-terminal CAAX box, whereas the other *Nematostella* complexin (nvCpx2) does not.

To test whether *Nematostella* complexins are functional and whether the function of complexin has been evolutionarily conserved, we constructed expression vectors encoding nvCpx1 and nvCpx2. We then examined whether *Nematostella* complexins could rescue the synaptic phenotype of complexin-deficient mouse neurons. Toward this goal, we suppressed endogenous complexin-1 and -2 expression by virally delivered shRNAs (referred to as the double knockdown [DKD]) [5,8] and examined rescue of the synaptic phenotype of complexin-deficient mouse neurons by *Nematostella* complexins. This approach was chosen because no tests of

Figure 1. Sequence alignments reveal that complexins are conserved from choanoflagellates to mammals.

A, B Complexin sequences from the indicated organisms (represented as organism name abbreviations followed by Cpx and optional numbers) are aligned in single-letter code (B). NCBI gene identification numbers follow the sequence names, except for two Ctenophora sequences (MICpx and PbCpx) that are derived from transcriptome assembly data. Color codes of shaded positions: yellow, mainly hydrophobic residues; blue, mainly positively charged residues; and gray, C-terminal CAAX boxes. Color codes of residues: blue, R and K; red, D and E. The four domains of complexins are indicated by the colored bar on top (A): cyan, N-terminal domain; gray, accessory α -helix; red, central α -helix; and green, C-terminal extended sequence. Sequence names are colored as follows: non-metazoans: magenta; basal metazoans: red; protostomes: blue; and deuterostomes: black. Organism name abbreviations are as follows: Aq, *Amphimedon queenslandica*; Bf, *Branchiostoma floridae*; Ce, *Caenorhabditis elegans*; Cm, *Callorhynchus milii*; Co, *Capsaspora owczarzewski*; Ci, *Ciona intestinalis*; Cg, *Crassostrea gigas*; Dr, *Danio rerio*; Dp, *Daphnia pulex*; Dm, *Drosophila melanogaster*; Fa, *Fonticula alba*; Gg, *Gallus gallus*; Hv, *Hydra vulgaris*; Lg, *Lottia gigantea*; Mb, *Monosiga brevicollis*; Mm, *Mus musculus*; Nv, *Nematostella vectensis*; Sk, *Saccoglossus kowalevskii*; Sp, *Strongylocentrotus purpuratus*; and Ta, *Trichoplax adhaerens*.

A Complexin Domain Map



B Complexin Sequence Alignment

Table of sequence alignments for various species (FaCpx, CoCpx, MbCpx, AqCpx, MLCpx, PbCpx, TaCpx, HVCpx, NvCpx1, NvCpx2, DpCpx, DmCpx, CeCpx1, CeCpx2, CgCpx, LgCpx, SpCpx1, SpCpx2, SkCpx, BfCpx, CiCpx, MmCpx1, GgCpx1, DRcpx1, CmCpx1, MmCpx2, GgCpx2, DrCpx2, DrCpx2l, CmCpx2, MmCpx3, GgCpx3, DrCpx3a, DrCpx3b, CmCpx3, MmCpx4, GgCpx4, DrCpx4, DrCpx4b, DrCpx4c, CmCpx4, CmCpx4l) across the four domains. Conserved residues are highlighted in yellow.

Figure 1.

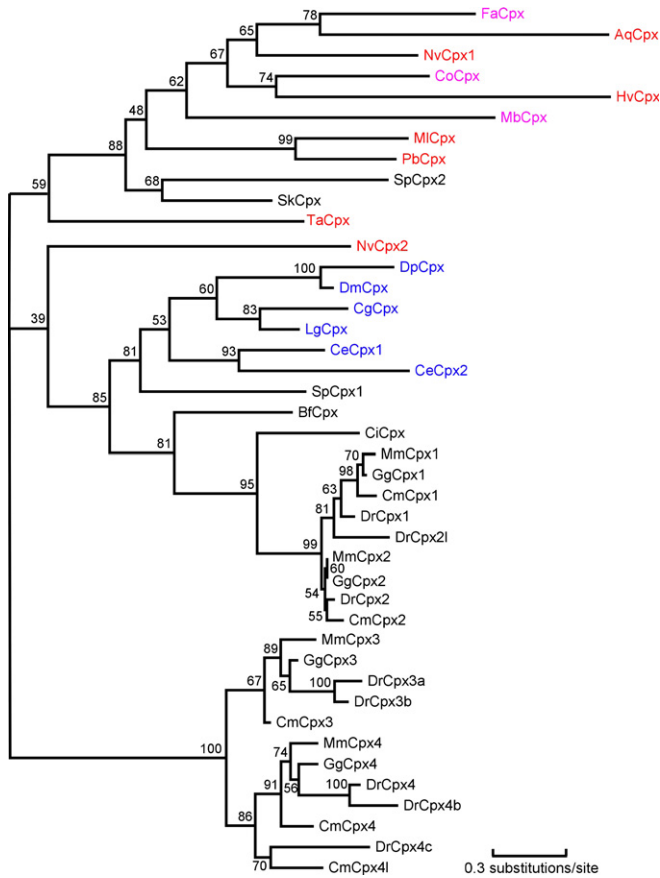


Figure 2. A phylogenetic tree of complexins.
 This tree was obtained by the MOLPHY package based on the alignment shown in Fig 1 (positions with 50% or more gaps were discarded before tree reconstruction). The coloring of sequence names is as follows: non-metazoans: magenta; basal metazoans: red; protostomes: blue; and deuterostomes: black.

complexin function in *Nematostella* itself are readily available—these metazoans do not have synapses that could be recorded from, and no functional assays of their morphologically identified neurosecretory cells have been reported—and because rescue of a loss-of-function over such a large evolutionary distance as that separating Cnidaria from rodents could be considered the most compelling evidence for functional similarity.

We first compared the nvCpx1 and nvCpx2 in parallel. To assess their potential clamping functions, we measured miniature excitatory postsynaptic currents (mEPSCs) in control mouse neurons and mouse neurons subjected to the complexin DKD without or with expression of either nvCpx1 or nvCpx2 (Fig 3). Consistent with previous data [5], we found that the complexin DKD significantly increased the mEPSC frequency, that is, unclamped mini release. This phenotype was ameliorated but not completely reversed by nvCpx1 and was even exacerbated by nvCpx2 (Fig 3A).

We then assessed the potential activator function of nvCpx1 and nvCpx2 by measuring action potential-evoked release (Fig 3B). We found that nvCpx1 fully rescued the impairment of evoked synaptic transmission in mouse neurons lacking complexins, whereas nvCpx2 had a much smaller rescue effect. Viewed together, these experiments suggest that nvCpx1 surprisingly may be a functional activator of release in mammalian synapses, whereas nvCpx2 is only weakly active, possibly because of expression impairments or evolutionary constraints. Thus, for further analyses, we focused on nvCpx1.

***Nematostella* complexin-1 activates neurotransmitter release similar to mammalian complexins**

In the next set of experiments, we asked how the function of NvCpx1 in neurotransmitter release quantitatively compared to that of a mammalian complexin. We examined the relative effects of rat Cpx1 (which is identical in amino acid sequence to mouse Cpx 1 [20]) and nvCpx1 on spontaneous mEPSCs in mouse cortical neurons

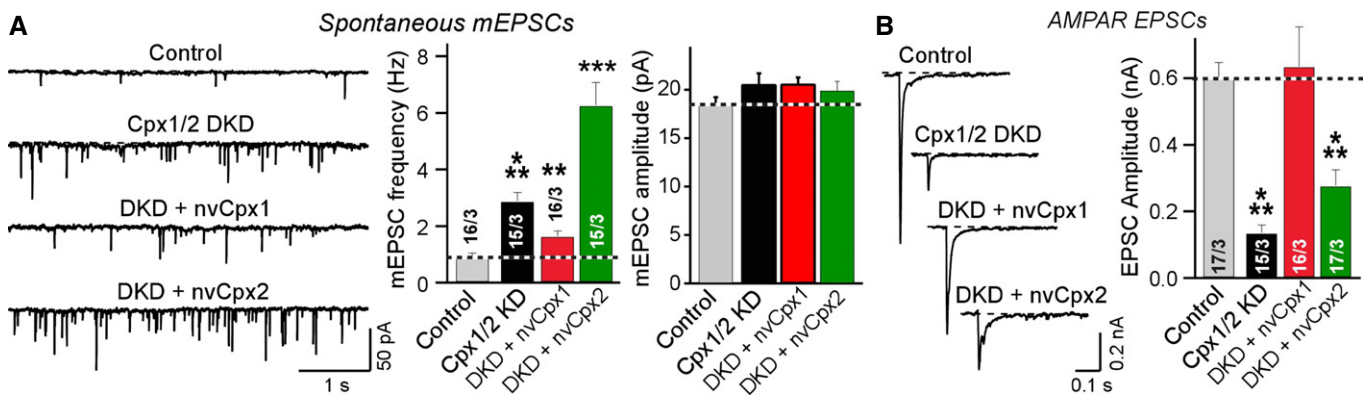


Figure 3. Testing rescue of complexin-deficient neurons with *Nematostella* complexin-1 (nvCpx1) or -2 (nvCpx2).
 A Rescue effects of *Nematostella* nvCpx1 and nvCpx2 on clamping spontaneous release in complexin-deficient mouse neurons. Panels show sample traces (left) and summary graphs of the frequency (center) and amplitude (right) of mEPSCs recorded in WT cortical neurons that were infected with a control lentivirus (Control) or a lentivirus expressing complexin shRNAs (Cpx1/2 DKD), without or with co-expression of *Nematostella* complexin-1 (nvCpx1) or -2 (nvCpx2).
 B Rescue of impaired evoked release in complexin-deficient mouse neurons by *Nematostella* nvCpx1 and nvCpx2. Panels show sample traces (left) and summary graphs of the amplitude of evoked AMPAR-mediated EPSCs that were induced by isolated action potentials.
 Data information: Data are means ± SEM; numbers of cells/independent cultures analyzed are listed in the bars. Statistical assessments were performed by Student's t-test comparing each condition (***P* < 0.01; ****P* < 0.001).

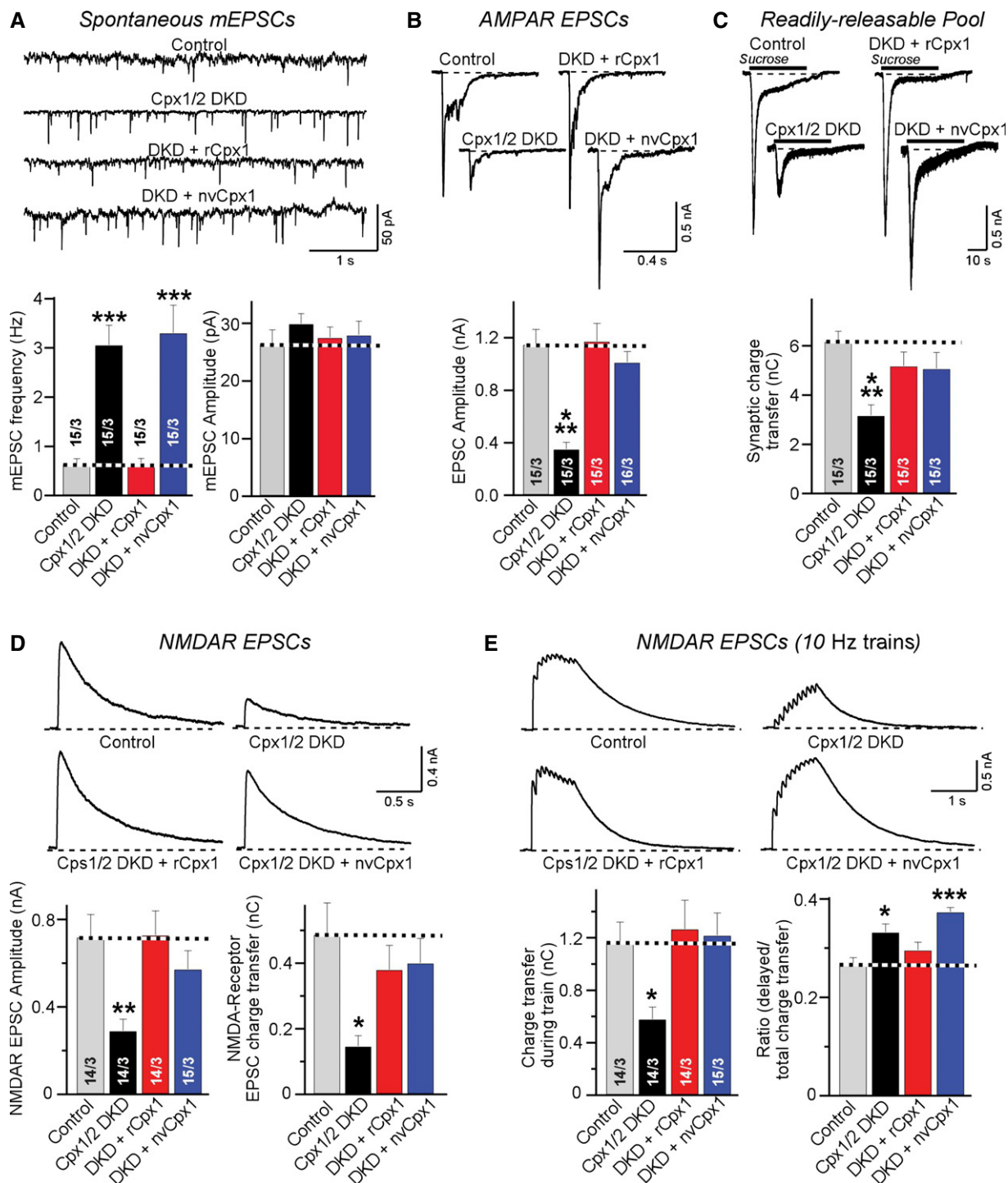


Figure 4. *Nematostella* complexin-1 functionally substitutes for mammalian complexins in enabling neurotransmitter release.

A Sample traces (top) and summary graphs (bottom) of mEPSCs recorded in WT cortical neurons that were infected with a control lentivirus (Control) or a lentivirus expressing complexin shRNAs (Cpx1/2 DKD), without or with co-expression of rat (rCpx1) or *Nematostella* complexin-1 (nvCpx1).

B Sample traces (top) and summary graphs (bottom) of evoked AMPAR-mediated EPSCs that were induced by isolated action potentials. EPSCs were monitored as described for (A).

C Sample traces (top) and summary graphs (bottom) of EPSCs evoked by 0.5 M sucrose, recorded from neurons as described for (A).

D Sample traces (top) and summary graphs (bottom) of NMDAR-mediated EPSCs evoked by isolated action potentials recorded from neurons as described for (A).

E Sample traces (top) and summary graphs of NMDAR-mediated EPSCs induced by a 10-Hz, 1-s stimulus train (bottom). Recordings were performed as in (A).

Data information: Data are means \pm SEM; numbers of cells/independent cultures analyzed are listed in the bars. Statistical assessments were performed by Student's *t*-test comparing each condition to control (* P < 0.05; ** P < 0.01; *** P < 0.001).

(Fig 4A). As expected, nvCpx1 neither aggravated nor rescued the unclamping of mini release in complexin DKD neurons, whereas rat Cpx1 fully rescued. We then examined the activating function of complexin. Complexins activate Ca^{2+} -triggered exocytosis by two sequential mechanisms: enhancement of synaptic vesicle priming and enabling of Ca^{2+} triggering by synaptotagmins. Only the former mechanism requires the C-terminal region of complexins [18]. To assess the activating function of nvCpx1, we measured action potential-evoked (i.e., Ca^{2+} -triggered) EPSCs, which are largely mediated by AMPA-type glutamate receptors (AMPA). We found that nvCpx1 fully rescued the ~3-fold decrease in evoked EPSC amplitude induced by the complexin DKD (Fig 4B). Moreover, we measured the size of the readily releasable pool (RRP) of vesicles, monitored as the synaptic charge transfer that occurs during an EPSC induced by hypertonic sucrose [21]. Again, we found that nvCpx1 rescued the ~2-fold decrease in the RRP induced by the complexin DKD (Fig 4C). These data indicate that nvCpx1 can substitute for mammalian complexin in activating both the Ca^{2+} triggering and the priming of vesicles for release, an unexpected finding given the evolutionary distance between *Nematostella* and mammals.

To corroborate this conclusion, we inquired whether nvCpx1 could also support neurotransmitter release during high-frequency stimulus trains. In high-density cultures of neurons, EPSCs induced by stimulus trains can only be monitored by measuring slower EPSCs mediated by NMDA-type glutamate receptors (NMDARs) with simultaneous inhibition of AMPARs that drive network activity [8]. We first confirmed that the rescue of evoked neurotransmitter release with nvCpx1 could also be observed in NMDAR-mediated EPSCs (Fig 4D). We then measured synaptic responses to a 10-Hz stimulus train monitored by NMDAR-mediated EPSCs. We observed that nvCpx1 was as competent as rat complexin-1 in rescuing the impairment of synaptic release induced by stimulus trains in complexin-deficient neurons (Fig 4E). We examined the release kinetics during the stimulus trains by plotting the cumulative charge transfer as a function of stimulus number, and detected no major differences in the kinetics of release during the stimulus train between neurons expressing rat or *Nematostella* complexin-1 (Fig EV2), suggesting that nvCpx1 fully supports release during the train. However, nvCpx1 did not reverse the slight increase in delayed release that is observed in complexin-deficient neurons (Fig 4E) [8]. Delayed release represents the release that continues after a stimulus train has ended [22]. The inability of nvCpx1 to reverse the increase in delayed

release in complexin-deficient neurons is consistent with its lack of a clamping function (Fig 4A), since the enhanced delayed release likely reflects, at least in part, an enhanced Ca^{2+} -dependent rate of spontaneous release [8].

***Nematostella* and mammalian complexin-1 exhibit similar functional domain architectures**

To determine whether nvCpx1 activates priming and Ca^{2+} triggering of release by mechanisms similar to those of mammalian complexin-1, we tested whether the N- and C-terminal regions of nvCpx1 were essential for Ca^{2+} triggering and priming of release, similar to the corresponding regions of mammalian complexin-1 [5,8,15,18].

When we examined spontaneous mEPSCs, we found that neither the N-terminal nor the C-terminal truncation of nvCpx1 endowed it with a clamping activity (Fig 5A), consistent with the initial results (Figs 3 and 4). We then measured Ca^{2+} -triggered release monitored by AMPAR-mediated EPSCs induced by single action potentials. We observed that the N- but not the C-terminal truncation blocked the Ca^{2+} -triggering function of nvCpx1 (Fig 5B), similar to mammalian complexin-1 [5,15,23]. However, the impairment in complexin-deficient neurons of sucrose-induced release, used to monitor synaptic vesicle priming, was not rescued by either C- or N-terminally truncated nvCpx1 (Fig 5C). Moreover, when we examined Ca^{2+} -triggered release in complexin-deficient neurons by monitoring NMDAR-mediated EPSCs, we also observed full rescue by C- but not by N-terminally truncated nvCpx1 (Fig 5D). Finally, release induced by high-frequency stimulus trains was also only rescued by C-terminally truncated nvCpx1, although as before nvCpx1 was unable to reverse the increase in delayed release induced by complexin DKD (Figs 5E and EV3). Thus, the activating and not the clamping function of complexin is evolutionarily conserved, demonstrating that there is no evolutionary switch in complexins from a primarily clamping to a primarily activating function, but that the activating functions of complexins are central to their role in all metazoans.

A possible reason for the lack of a clamping activity of nvCpx1 in our experiments may be its C-terminal isoprenylation sequence. Mammalian complexin-3 and -4 also contain such a C-terminal sequence and lack clamping activity [18,24], and in *Drosophila* and *C. elegans*, this sequence has been implicated in the clamping capability of complexins [13,14,25]. To test this possibility, we abolished

Figure 5. Functional domain organization of *Nematostella* complexin-1 is similar to that of mammalian complexins.

- A Sample traces (top) and summary graphs (bottom) of mEPSCs, recorded in cortical neurons that were infected with a control lentivirus (Control) or lentiviruses expressing complexin shRNAs (Cpx DKD) without or with co-expression of N- (nvCpx²⁸⁻¹²¹) or C-terminally truncated *Nematostella* complexin-1 (nvCpx¹⁻⁹⁰).
- B Sample traces (top) and summary graphs (bottom) of action potential-evoked AMPAR-mediated EPSCs monitored in neurons as described for (A).
- C Sample traces (top) and summary graphs of AMPAR-mediated EPSCs evoked by 0.5 M sucrose (bottom), recorded as described for (A).
- D Sample traces (top) and summary graphs (bottom) of NMDAR-mediated EPSCs evoked by isolated action potentials recorded as described for (A).
- E Sample traces (top) and summary graphs (bottom) of NMDAR-mediated EPSCs evoked by action potential trains (10 Hz for 1 s) recorded as described for (A).
- F Sample traces (top) and summary graphs (bottom) of mEPSCs, recorded in WT cortical neurons that were infected with a control lentivirus (Control) or a lentivirus expressing complexin shRNAs (Cpx DKD) without or with rescue with mutant *Nematostella* complexin-1 in which the C-terminal cysteine that is presumably isoprenylated (Fig 1A) was converted to a serine (nvCpx^{c1.185}).
- G Sample traces (top) and summary graphs (bottom) of action potential-evoked AMPAR-mediated EPSCs monitored as described for (A).
- H Sample traces (top) and summary graphs of EPSCs evoked by 0.5 M sucrose (bottom), recorded as described for (A).

Data information: Data are means \pm SEM; numbers of cells/independent cultures analyzed are listed in the bars. Statistical assessments were performed by Student's t-test comparing each condition to control (* P < 0.05; ** P < 0.01; *** P < 0.001).

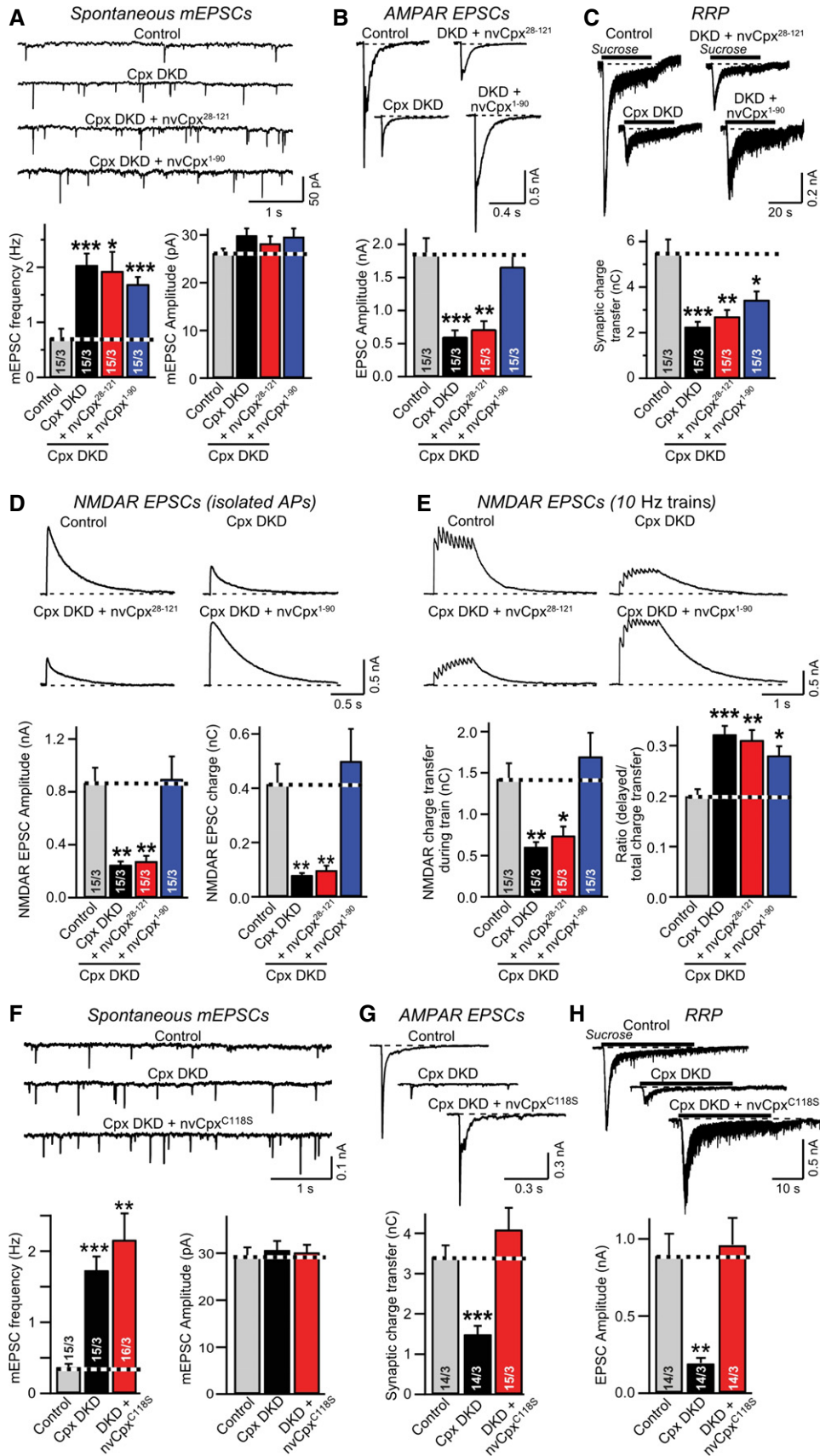


Figure 5.

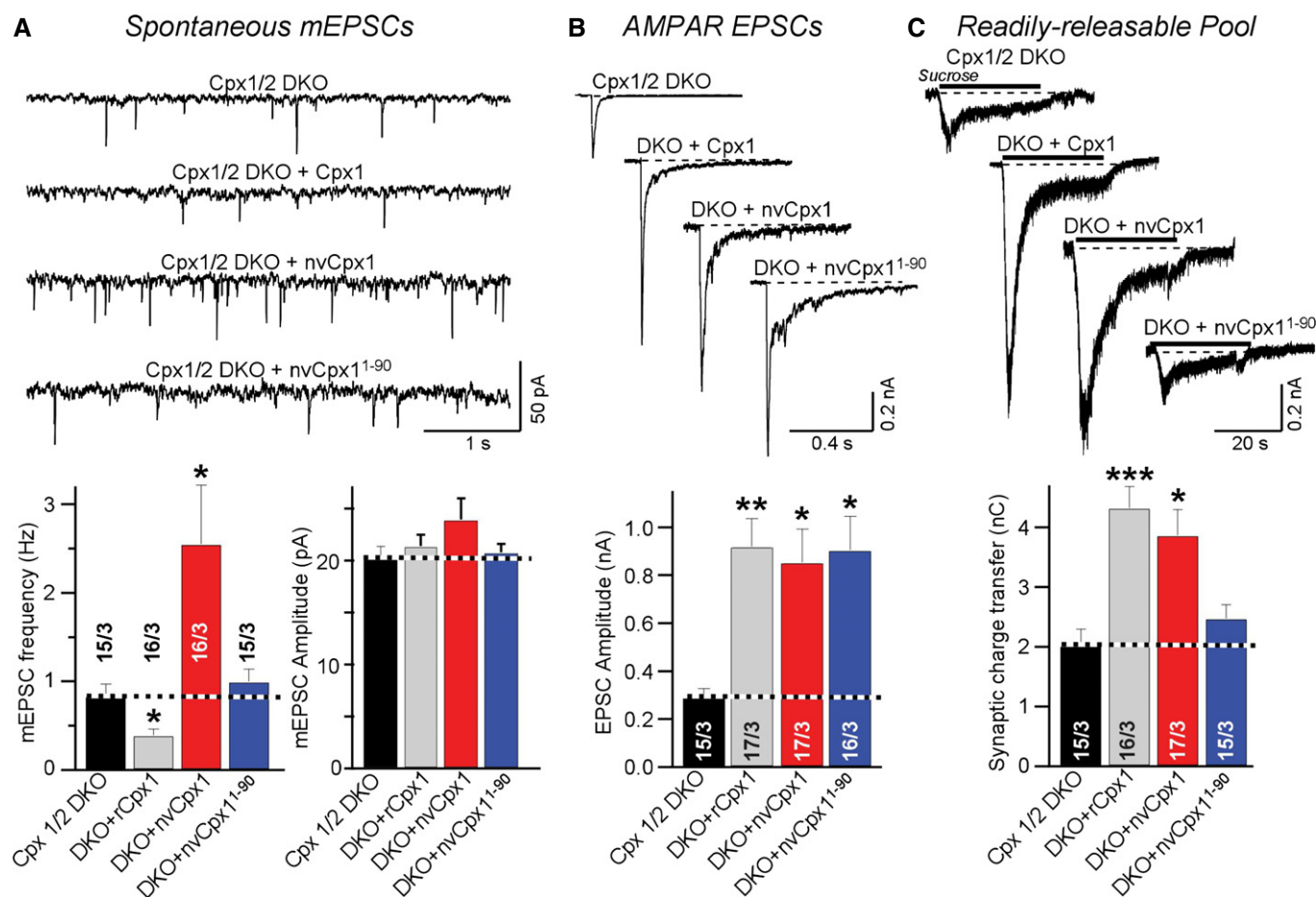


Figure 6. *Nematostella* complexin-1 rescues evoked neurotransmitter release in cortical neurons cultured from complexin-1/2 double KO mice.

A Sample traces (top) and summary graphs (bottom) of mEPSCs, recorded in cortical neurons cultured from complexin-1/2 double KO mice [23]. Neurons were infected with a control lentivirus (Cpx1/2 DKO) or a lentivirus expressing wild-type rat complexin-1 (rCpx1), wild-type *Nematostella* complexin-1 (nvCpx1), or C-terminally truncated *Nematostella* complexin-1 (nvCpx1¹⁻⁹⁰).

B Sample traces (top) and summary graphs (bottom) of action potential-evoked AMPAR-mediated EPSCs monitored as described for (A).

C Sample traces (top) and summary graphs of EPSCs evoked by 0.5 M sucrose (bottom), recorded as described for (A).

Data information: Data are means \pm SEM; numbers of cells/independent cultures analyzed are listed in the bars. Statistical assessments were performed by Student's *t*-test comparing each condition to control (* P < 0.05; ** P < 0.01; *** P < 0.001).

the isoprenylation potential of nvCpx1 by mutating the putative isoprenylated cysteine residue (C118) to a serine (nvCpx1^{C118S}).

We then tested whether lack of isoprenylation endows nvCpx1 with a clamping ability, but observed that the mutation did not have any effect in mEPSCs (Fig 5F). Moreover, nvCpx1^{C118S} was still capable of activating Ca²⁺ triggering and priming of synaptic vesicle exocytosis (Fig 5G and H), similar to the lack of an effect we observed when we converted mammalian complexin-1 into an isoprenylated protein [26]. Thus, C-terminal isoprenylation of nvCpx1 is not functionally essential. Overall, these results indicate that nvCpx1 acts by a similar mechanism as mammalian complexins [26].

***Nematostella* complexin-1 also rescues release in complexin-1/2 double KO (DKO) neurons**

The complexin DKD approach in our experiments, although well validated, may raise concerns because of potential off-target effects

[27]. In a direct comparison, we previously found that the DKD and the double KO of complexin-1 and -2 in mouse neurons caused identical priming and Ca²⁺-triggering phenotypes, but distinct clamping phenotypes and compensatory changes in mRNA levels of complexin-3 and -4 [26]. We thus sought to further validate our results with nvCpx1 in complexin DKD neurons using complexin DKO neurons [28].

Consistent with earlier results [26], we found a significant but small increase in spontaneous mEPSC frequency induced by the complexin-1/-2 DKO in cortical neurons that was rescued by the expression of rat complexin-1 (Fig 6A). Expression of nvCpx1, however, caused a large increase in mEPSC frequency, which may be explained by earlier observations showing that overexpression of mutant complexins that are unable to clamp spontaneous release causes an unclamping of mEPSC release by a dominant-negative mechanism [26]. Strikingly, this unclamping was abolished by deletion of the C-terminal regions from nvCpx1 (Fig 6A). Moreover,

full-length as well as C-terminally truncated nvCpx1 was as effective as mammalian complexin-1 in increasing evoked EPSCs in the DKO neurons, confirming that nvCpx1 is fully capable of activating release (Fig 6B). Finally, as in the DKD neurons, full-length but not C-terminally truncated nvCpx1 was as active as mammalian complexin-1 in increasing the RRP size in complexin-1/-2 DKO neurons (Fig 6C). Together, these experiments confirm that in DKO neurons, nvCpx1 is fully competent to replace the complexin activator functions in mammalian neurons.

Summary

Overall, our experiments suggest two major conclusions. First, complexins likely predate metazoan evolution and may have a general role in membrane traffic. Their emergence prior to that of neurosecretory cells is consistent with the observation of complexins in non-neuronal mammalian cells [20], and suggests that complexins are fundamental components of all types of regulated exocytosis. Second, the function of complexins in the core machinery of neurotransmitter release appears to be conserved throughout metazoan evolution. To the best of our knowledge, our study reports the first functional expression of a cnidarian protein in mammalian neurons, and the first demonstration that cnidarian exocytosis operates by fundamentally identical molecular mechanisms as mammals. The fact that *Nematostella* complexin can replace mouse complexin in activating neurotransmitter release indicates that the fundamental function of complexin consists of an activating role, a role that is essential for preparing the fusion machinery for fast regulated exocytosis.

Materials and Methods

Bioinformatics

PSI-BLAST [ref: PubMed 9254694] was used to search for complexins against the nr database of NCBI, with the human complexin-3 as the initial query (e-value cutoff: 0.001). Found homologs were clustered using BLASTCLUST, and one representative sequence was selected from each cluster and used as query for further PSI-BLAST iterations. Multiple sequence alignment of select complexins from major lineages of Metazoa and non-metazoans was made by PROMALS3D (PMID: 18287115), followed by manual adjustment. The MOLPHY package [29] was used for phylogenetic reconstruction of these proteins based on the alignment, with positions containing 50% or more gap characters removed. The JTT amino acid substitution model [30] was used in MOLPHY. The local estimates of bootstrap percentages (shown next to branch points) were obtained by the REL method [31] (-R option in the ProtML program of MOLPHY).

Neuronal cultures and lentiviruses preparation

Neuronal cultures were obtained from wild-type (WT) or complexin-1/2 double KO mice [26,28] as described [8]. Lentiviral expression vectors and three helper plasmids (pRSV-REV, pMDLg/pRRE, and pVSVG) were co-transfected into HEK293 cells (ATCC, VA), and the viruses were collected 48 hr after transfection [5]. All

steps were performed under level II biosafety conditions. Neurons were infected with lentiviruses at DIV4 and analyzed at DIV14-16. All mouse procedures used were approved by Stanford Institutional Review Boards.

Plasmid construction

Constructs encoding WT *Nematostella* complexin-1 (nvCpx1) and mutants thereof (the N- (nvCpx²⁸⁻¹²¹) and C-terminal truncations (nvCpx¹⁻⁹⁰) and the C-terminal cysteine substitution (nvCpx^{C118S})) were generated by gene synthesis and were cloned downstream of the human ubiquitin promoter in the L309 lentiviral vector [5].

Electrophysiological recordings

Electrophysiological recordings were performed in whole-cell patch-clamp mode. Synaptic currents were monitored with a Multiclamp 700A amplifier (Molecular Devices). The frequency, duration, and magnitude of the extracellular stimulus were controlled with a Model 2100 Isolated Pulse Stimulator (A-M Systems) synchronized with Clampex 10 data acquisition software (Molecular Devices). AMPA receptor- and NMDA receptor-mediated EPSCs (recorded at a holding potential of -70 mV and $+40$ mV, respectively) were isolated pharmacologically with D-APV and picrotoxin, and with CNQX, picrotoxin, and glycine, respectively. Spontaneous mEPSCs were monitored in the presence of tetrodotoxin (TTX). Sucrose-evoked release was triggered by a 30-s application of 0.5 M sucrose with D-APV, picrotoxin, and TTX, puffed by Picospritzer III (Parker).

Statistical analyses

Statistical analyses were performed with Student's *t*-tests or two-way ANOVA (for Figs EV1–3) comparing test to control samples analyzed in the same experiments.

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Author contributions

XY, YJK-W, and TB planned and performed the experiments, analyzed the data, and wrote the paper; JP and NVG performed bioinformatics analyses; and TCS analyzed the data and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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