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The non-neuronal syntaxin SYN-1 regulates defecation behavior and neural activity in *C. elegans* through interaction with the Munc13-like protein AEX-1

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Abstract

We have previously shown that the AEX-1 protein, which is expressed in postsynaptic muscles, retrogradely regulates presynaptic neural activity at the *Caenorhabditis elegans* neuromuscular junctions. AEX-1 is similar to vertebrate Munc13-4 protein, suggesting a function for vesicle exocytosis from a kind of cells. Compared to emerging evidences of the role of Munc13 proteins in synaptic vesicle release, however, the precise mechanism for vesicle exocytosis by AEX-1 and Munc13-4 is little understood. Here we have identified SYN-1 as a candidate molecule of AEX-1-dependent vesicle exocytosis from non-neuronal cells. The *syn-1* gene encodes a *C. elegans* syntaxin, which is distantly related to the neuronal syntaxin UNC-64. The *syn-1* gene is predominantly expressed in non-neuronal tissues and genetically interacts with *aex-1* for presynaptic activity. However, the two proteins did not interact physically in our yeast two-hybrid system and mutational SYN-1 did not bypass the requirement of AEX-1 for the behavioral defects in *aex-1* mutants, whereas mutant UNC-64 does in *unc-13* mutants. These results suggest that a novel molecular interaction between the AEX-1 and syntaxin may regulate vesicle exocytosis for retrograde signal release.

Keywords

Vesicle exocytosis; Syntaxin; UNC-13; Retrograde signaling; *C. elegans*

Synapse formation, maintenance, and plasticity require reciprocally coordinated signaling between pre- and postsynaptic cells. Retrograde signaling from postsynaptic cells to presynaptic neurons has been implicated in synaptogenesis and activity-dependent synaptic plasticity [1]. Previous studies have shown that several molecules are secreted from postsynaptic cells and retrogradely regulate presynaptic neuronal development or activity [2, 3]. However, the regulatory machinery of retrograde signal release is still largely unknown. What kinds of molecules regulate release of the retrograde signals, where they are released

from, and how their release is regulated, either in an activity dependent- or activity independent-manner?

We have previously identified the AEX-1 and AEX-5 proteins, which regulate retrograde signaling at the NMJ of *Caenorhabditis elegans* [4]. Both *aex-1* and *aex-5* mutants show several defects including defecation defects called Aex phenotype and reduced transmitter release from presynaptic terminals [4,5]. In both mutants, presynaptic defects are retained by the muscle-specific expression of each gene whereas defecation defects are retained by the intestine-specific expression of each gene, suggesting that any signals from non-neuronal tissues retrogradely affect neural activity. AEX-1 protein is similar to vertebrate Munc13-4 protein, which is a member of the UNC-13 protein family and presumably regulates vesicle exocytosis from the cells [6]. AEX-5 is a subtilisin-like prohormone convertase that acts as an enzyme for peptide maturation [7]. These results strongly suggest that peptidic signals catalyzed by AEX-5 are released from muscles and the intestine through vesicle exocytosis dependent on AEX-1. However, the signal itself is not yet understood, nor are the other molecules acting for vesicle exocytosis from postsynaptic tissues.

Unlike other members of the UNC-13 protein family, AEX-1 and Munc13-4 proteins do not contain a long N-terminal region followed by a C1 domain, which binds to phorbol esters and DAG [6]. This may suggest a distinct mechanism for AEX-1/Munc13-4 proteins in vesicle exocytosis. Recent evidences indicate that UNC-13/Munc13-1 proteins can interact with the presynaptic SNARE protein syntaxin, which functions for the target SNARE protein [8-11]. Munc13-4 has been also shown to function in vesicle exocytosis from several secreted cells, and mutations cause immunological defects called FHLH [12]. Interestingly, mutations in Syntaxin11 also cause FHLH, suggesting that Munc13-4 and Syntaxin11 may act in the same exocytic pathway at immunological synapses [13]. However, the functional significance of the Munc13-4/Syntaxin11 interaction has not been tested directly. In this study, to understand the molecular mechanism for AEX-1-dependent vesicle release in retrograde signaling, we performed genetic screening to isolate mutants showing the same defecation defects with *aex-1* mutants, and we examined the interaction between the isolated mutants and *aex-1*.

Materials and methods

Strains and culturing

Wild-type strains were *C. elegans* variety Bristol strain, N2. Nematodes were grown at 20 °C on standard Nematode Growth Medium, seeded with bacterial OP50. The *aex-1(sa9); syn-1(tg94)* double mutant strain was constructed using standard genetic methods, and both mutations were confirmed by direct sequencing.

Isolation, mapping, and cloning of the *tg94* mutant allele

Synchronized N2 hermaphrodites (L4) were mutagenized in 50 mM EMS for 4 h at room temperature. After two generations, defecation-defective mutants were isolated by direct observation. Mutant animals were backcrossed to N2 animals at least five times. In the newly isolated mutants, the *tg94* allele was successfully mapped between cosmids C31H2 and F35C8 on chromosome X using a standard SNP-mapping method. A full-length *syn-1* cDNA was amplified from the yk745c4 clone (a gift from Dr. Kohara), and the full-length sequence was confirmed by sequencing both the yk clone and RT-PCR products.

Behavioral assays

Defecation assays were performed as previously described [4]. Aldicarb and levamisole tests were performed as previously described [4]. All drugs tests were blind and were repeated five times in each genotype.

Molecular biology

To examine the expression pattern of the *syn-1* gene, a 6.3-kb PCR fragment (from the 3.0 kb promoter region of the former gene C26B9.1 to the start codon of *syn-1*), was sub-cloned in the pPD95.77 GFP vector, generating pDK129. A rescuing construct, pDK130, was generated by subcloning an 11-kb fragment containing the 6.3-kb 5' upstream region, the 3.3-kb full-length *syn-1* coding region, and the 1.9-kb 3' downstream region of *syn-1*, in pPD95.77. To generate a GFP::SYN-1 translational fusion construct, pDK320, a GFP fragment was inserted in frame with the *syn-1* ATG start codon of the pDK130 plasmid.

Plasmids for tissue-specific expression of *syn-1* were constructed as follows. Full-length *syn-1* cDNA was subcloned into the pPD96.52. The following promoter sequences were inserted for tissue-specific expression: intestine: *elt-2* (5.1-kb); neuron: *unc-119* (1.2-kb); and muscle: *myo-3* (2.5-kb). A full-length *unc-64* cDNA was amplified by RT-PCR and was substituted with the *syn-1* cDNA sequence to generate a *Pelt-2::UNC-64* plasmid.

To generate the open-formed SYN-1 plasmid, both the 177th Ile and 178th Glu of the *syn-1* cDNA were converted into Ala. The mutational cDNA was amplified using primer sets with corresponding mutations and was substituted with the wild-type cDNA in the *Pelt-2::SYN-1* plasmid. All plasmids were sequenced to check mutations, and all primers used in this study are available upon request.

Results

Isolation of the defecation-defective mutant *syn-1*

aex-1 mutants show severe defecation-defective phenotypes, which specifically lack aBoc and Exp steps in the three defecation muscle contractions [5]. To identify other signaling molecules which potentially interact with AEX-1, we performed a large scale genetic screening to isolate the mutants that show similar defecation defects, as observed in *aex-1* mutants. From this screening we successfully isolated several Aex mutants and focused on the *tg94* mutant allele. In the *tg94* mutant, the frequency of the aBoc and Exp steps in a defecation cycle was reduced to 20% (Fig. 1A), while the pBoc step was normal. This defecation defect was slightly milder than that in *aex-1*.

Cloning of *syn-1*

To understand the molecular characteristics of the *tg94* allele, we performed genetic mapping to identify the gene responsible for defecation defects in the *tg94* allele. The *tg94* allele was successfully mapped between cosmids C31H2 and F35C8 on chromosome X (Supplementary Fig. 1A). By directly sequencing candidate genes in this region, a missense mutation (G to A substitution) was found in the predicted exon 4 of the F35C8.4 (*syn-1*) gene (Fig. 1C). To confirm that *tg94* is an allele of *syn-1*, an 11-kb genomic DNA fragment (see Fig. 1C) was introduced into the *tg94* mutant animals. The resulting transgenic animals showed complete rescue of the defecation defects in the *tg94* mutant (Fig. 1A). Furthermore, the feeding RNAi-treatment using a *syn-1* double-strand RNA caused an Aex phenotype similar to the *tg94* mutant (data not shown). From these results, we conclude that *tg94* is an allele of *syn-1*, and that *syn-1* is responsible for the defecation behavior.

We cloned the full-length *syn-1* cDNA from yk745c4 and fully sequenced it. *syn-1* encodes a syntaxin which acts as a target SNARE in vesicle fusion. The SYN-1 protein contains all of the conserved domains in the syntaxin family: the putative syntaxin amino-terminal (SynN), the target SNARE (t-SNARE), and the transmembrane (TM) domain (Supplementary Fig. 1C). The SynN domain is implicated in the regulation of the SNARE-complex assembly by folding to the t-SNARE domain through interaction with several regulatory proteins. The t-SNARE

domain is required for SNARE formation with other SNARE proteins. The *tg94* mutation led to the substitution of a glycine for glutamate residue at position 123, where it is involved in the SynN domain (Supplementary Fig. 1C). Therefore, it is possible that the *tg94* mutation may affect SNARE-complex assembly with other SNARE proteins. In the human syntaxins, Syntaxin11 looks to be phylogenically most similar to SYN-1, while neuronal Syntaxin1A is distantly related to SYN-1 (Supplementary Fig. 1B). In the *C. elegans* genome, eight syntaxin genes are found [14], and SYN-1 is closer to SYN-4 and SYN-2 than to the neuronal syntaxin UNC-64, which has been well analyzed due to its role in synaptic vesicle release [15]. Thus, SYN-1 is probably a plasma membrane-type syntaxin distantly related to neuronal syntaxins in *C. elegans* and the vertebrates.

syn-1 functions in the intestine for defecation behavior

aex-1 is expressed in the intestine and body-wall muscles, and these expression patterns correspond with the mutant phenotypes. To understand the relationship between *aex-1* and *syn-1* more clearly, we examined which types of cells express *syn-1*. Since there exists a yk clone which includes a *syn-1* transcript fused with the former gene's transcript (C26B9.1), we generated a GFP reporter construct fully containing the 3.0 kb upstream region and the coding region of the C26B9.1 gene (pDK129, Fig. 1C). GFP expression was observed in the intestine and in most muscle cells, such as the pharyngeal, body-wall, vulval and enteric muscles (Fig. 2A and B). In contrast to the pan-neuronal expression of UNC-64 [15], a small number of neuronal cells in the head region expressed *syn-1*. None of these neurons were AVL nor DVB neurons, which regulate aBoc and Exp steps in the defecation behavior [16]. Similar expression patterns with *aex-1* may indicate the functional relationship between the two proteins.

To know where SYN-1 is localized in the cells, we generated a translational fusion construct in which GFP was inserted just after the ATG start codon of SYN-1 (pDK320, Fig. 1C) and introduced it in worms. This fusion construct successfully rescued the defecation defects in the *syn-1* mutant, suggesting that the fusion protein is functional and that the localization pattern is meaningful. GFP signals were observed at the margin of the cells expressing the fusion proteins, indicating that fusion proteins localize at or near the plasma membrane (Fig. 2C and D). These results support the idea that SYN-1 is a plasma membrane syntaxin for the regulation of vesicle exocytosis from the cells.

Next, to determine whether SYN-1 in the intestine also regulates defecation behavior like AEX-1, we examined the tissue-specific rescue ability of the *syn-1* mutant by expressing SYN-1 under the control of tissue-specific promoters. Neither muscle-specific nor neuron-specific expression of SYN-1 restored normal defecation. However, intestine-specific expression completely rescued defecation defects in the *syn-1* mutant (Fig. 2E). Thus, we conclude that SYN-1 regulates the defecation motor steps, aBoc and Exp, in the intestine, presumably functioning for vesicle exocytosis.

The SynN domain is required for SYN-1 function in defecation behavior

Neuronal syntaxin UNC-64 is also expressed in the intestine, and hypomorphic *unc-64* mutants show weak defects in defecation [15]. To more precisely understand the function of the two syntax-ins in the intestine, we tested whether overexpression of UNC-64 could replace SYN-1 function in defecation. Intestine-specific expression of UNC-64 did not rescue the defecation defect in the *syn-1* mutant (Fig. 3B), suggesting that SYN-1 has a specific function in defecation behavior. To further identify any domains in SYN-1 that are essential for defecation, we generated several chimeric constructs between SYN-1 and UNC-64 and examined rescue ability of defecation defects (Fig. 3). In these chimeric proteins, only the proteins having the SynN domain from SYN-1 clearly rescued the defects. No other constructs had any rescue abilities at all. These data strongly suggest that SYN-1 specifically controls the defecation

behavior, presumably through interaction between the SynN domain and other regulatory molecules, such as AEX-1.

***syn-1* and *aex-1* interact genetically, but the protein interaction is not same with presynaptic protein interaction**

Similar defecation defects in *syn-1* and *aex-1* mutants, similar expression patterns of the genes, and the same rescue ability for defecation defects in the intestine, all of these results suggest that the two proteins should interact with each other and cooperatively regulate vesicle release from the intestine, similarly to the function of UNC-13 and UNC-64 in presynaptic vesicle release. We examined both genetic and physical interaction between SYN-1 and AEX-1 by testing double mutant phenotypes and by using a yeast two-hybrid assay.

Defecation defects in the double mutant animals were almost the same as with *aex-1* single mutants (Fig. 1A), but these quite severe phenotypes did not illustrate genetic interaction between them. So we focused on another phenotype in *aex-1* mutants, the decreased sensitivity to aldicarb, an inhibitor of acetylcholinesterase [4]. The *aex-1* mutant animals showed mild resistance to 0.5 mM aldicarb compared with wild-type animals. *syn-1(tg94)* mutant animals, however, did not show any resistance to aldicarb (Fig. 1B). On the other hands, the *aex-1*; *syn-1* double mutant animals showed more severe resistance compared to the *aex-1* single mutant (Fig. 1B). Similar results were obtained using different concentrations of aldicarb (data not shown). The fact that the null phenotype of the *aex-1(sa9)* mutant was affected more severely by introducing the second *tg94* mutation which shows the wild-type phenotype, indicates that *syn-1(tg94)* act as a genetic enhancer of *aex-1*, and that genetic interaction exists between these genes. The *syn-1(tg94)* allele may not be a simple loss-of-functional mutation. Furthermore, the double mutant animals were not resistant an acetylcholine agonist, levamisole (Supplementary Fig. 2), suggesting that presynaptic transmitter release is decreased in the mutants.

According to the genetic interaction between *aex-1* and *syn-1*, we examined physical interaction between the SYN-1 and AEX-1 proteins using a yeast two-hybrid system. Surprisingly, we did not detect any positive interaction between each full-length, N-terminal or C-terminal peptide of the proteins in this assays (Supplementary Fig. 3A). Furthermore, based on clear positive interaction between the UNC-13 MHD2 domain and UNC-64 short N-terminal domain [10], interaction between the corresponding peptides from AEX-1 or SYN-1 was examined (Supplementary Fig. 3B). No positive interaction was observed between those peptides. These results imply that these two proteins probably act in the same vesicle release machinery, but may not interact directly for their molecular functions.

The open-form of SYN-1 does not bypass the requirement of AEX-1 for defecation behavior

It may be possible, however, that yeast two-hybrid analysis does not precisely reflect the interaction between the two proteins in the *C. elegans* cells. Previous studies report that syntaxin can adopt two configurations, a closed state and an open state. Only the open conformation is able to form SNARE complex with other SNARE proteins, such as synaptobrevin and SNAP-25 [9]. It has also been revealed that UNC-13 binds to the N-terminus of UNC-64 and promotes the open configuration of UNC-64 [10]. For this reason, the open conformation of UNC-64 can bypass the requirement of UNC-13 for vesicle docking or priming and retains phenotypes nearly identical to the wild-type in *unc-13* mutants [11]. Because AEX-1 and SYN-1 belong to the Munc13 and syntaxin families, respectively, we hypothesized that if the open configuration of SYN-1 could bypass the AEX-1 function for defecation, AEX-1 should interact with SYN-1 for its conformational change. To examine this hypothesis, we generated an open-form of SYN-1 by introducing corresponding mutations (I177A and E178A). These amino acids are conserved in human Syntaxin11, and are similar

to both UNC-64 and human syntaxin1A (Supplementary Fig. 1C). First, we expressed this open SYN-1 in the intestine of *syn-1* mutants. In the resulting transgenic animals, defecation defects were only rescued partially: the average frequency of Exp per defecation cycle was half of the almost complete rescue by the wild-type SYN-1 (Fig. 4). This result is not consistent with the complete rescue of all defective phenotypes in *unc-13* mutants by open-form UNC-64 [11]. Next, we introduced the open SYN-1 into the *aex-1(sa9)* mutant to examine whether the open SYN-1 could bypass the requirement for AEX-1 function in defecation. However, no rescue ability was observed in the resulting *aex-1; Open SYN-1* transgenic animals (Fig. 4). These results suggest that mutated open-form SYN-1 may have a different molecular property than the almost wild-type property of open-form UNC-64, and that molecular interaction between AEX-1 and SYN-1 may not be the same as that between UNC-13 and UNC-64.

Discussion

In this study, we have found a possible interaction between SYN-1 and AEX-1, which functions for both defecation behavior and retrograde signaling at the NMJ in *C. elegans*. These two molecules should act in the same molecular pathway for a kind of vesicle release from the intestine and/or muscles. However, no direct interaction between two proteins in yeast two-hybrid assays and no rescuing activity of the open-form SYN-1 in the *aex-1* mutant background raise a possibility that these two proteins probably have a distinct molecular property compared to the presynaptic UNC-13 and UNC-64 interaction. This does not exclude direct interaction or molecular complex between AEX-1 and SYN-1 in the *C. elegans* cells. However, considering the strong positive interaction between UNC-13 and UNC-64 in the same yeast two-hybrid condition, AEX-1 may require other factors to interact with SYN-1 for its conformational change in vivo. Alternative most likely explanation is that, in the intestine and/or muscles, AEX-1 does not facilitate the conformational change of SYN-1 protein, and that SYN-1 does not change its configuration from 'close' to 'open' for functional SNARE formation with other SNARE molecules. This open configuration of syntaxin-independent vesicle release may be specific to the secretion machinery for peptides or hormone-like molecules from several kinds of cells. In the AEX-1-dependent retrograde signaling, the released molecule should be a peptide. Similar molecular interaction in Munc13-4/Syntaxin11 can be speculated at the immunological synapses between a cytotoxic T lymphocyte and its target cell. At this synapses, released molecule is perforin which kills target cells [13].

The increased resistance to aldicarb in the double mutant was quite surprising because the *syn-1(tg94)* single mutant animals showed normal response. This indicates that the two genes probably do not function in a parallel or distinct pathway because their response was not an additive of both phenotypes. Thus, it is highly possible that *syn-1* functions in the same genetic pathway as *aex-1*, and that the *syn-1* mutation synergistically increases resistance to aldicarb in the *aex-1* mutant background as a genetic enhancer. This means that SYN-1 and AEX-1 cooperatively act in the same vesicle exocytosis cascade, but that SYN-1 may have an additional role other than the AEX-1-requiring step in vesicle exocytosis. Is *syn-1* really involved in *aex-1*-dependent retrograde signaling at the NMJ? The increased resistance to aldicarb in the double mutant probably results from presynaptic defects, because the response to levamisole was not decreased at all. Expression patterns of both genes support the postsynaptic functions of both proteins. Thus, we believe that the SYN-1 should regulate presynaptic neuronal activity from postsynaptic muscles. Isolation of the null allele of *syn-1* and direct observation of presynaptic defects, such as diffuse localization of the UNC-13 protein at the presynaptic terminals in *aex-1* mutants, will provide direct evidence that SYN-1 is a component of SNARE for the regulation of retrograde signal release from postsynaptic cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Phenotypes of *syn-1* mutants and cloning of *syn-1*. (A) Defecation defects in *syn-1*, *aex-1*, and their double mutants. Frequency of enteric muscle contraction (Exp) in defecation cycles is shown. 'SYN-1(+)' indicates the transgenic line containing *Psyn-1::syn-1* genomic DNA. At least 10 animals were observed for each genotype. Error bars indicate the standard error of mean (SEM). (B) Sensitivity to 0.5 mM aldicarb. The double mutant showed an increased resistance compared to the *aex-1* single mutant animals. (C) The gene structure of F35C8.4 (*syn-1*). Filled boxes indicate coding exons; gray boxes indicate untranslated regions of the *syn-1* gene. Arrows indicate the position of mutation in the *tg94* allele. Bars under the gene

structure indicate genomic DNA regions used to generate rescue- or expression-analysis plasmids.

Fig. 2.

SYN-1 is predominantly expressed in non-neuronal tissues and is localized on the plasma membrane. (A, B) Expression patterns of the *syn-1* gene. (A) Head region. *syn-1* is expressed in pharyngeal muscles (arrow) and body-wall muscles (arrowhead). (B) Tail region. GFP expression is observed in the intestine (thin arrow) and enteric muscles including the intestinal muscle (closed arrowhead), the anal sphincter (open arrowhead) and anal depressor muscle (thick arrow). (C, D) Subcellular localization of the GFP:: SYN-1 fusion protein. (C) Body-wall muscle; (D) intestine. The fusion protein localized around the cell margin (arrowheads). Scale bars, 50 μ m. (E) SYN-1 functions in the intestine for defecation behavior. The promoters

used for tissue-specific expression of *syn-1* cDNA are as follows: *elt-2*; the intestine, *myo-3*; body-wall muscles, *unc-119*; pan-neuronal.

Fig. 3.

The N-terminal region of SYN-1 is required for SYN-1 function in defecation behavior. (A) Schematic drawings of chimeric proteins between SYN-1 and UNC-64. Black indicates the SYN-1 amino acid region; white indicates the UNC-64 region. Numbers under the bars correspond to the amino acid of SYN-1. (B) Rescues of the defecation defects by chimeric proteins. The chimeric proteins containing the SynN domain from the SYN-1 protein significantly rescued defecation defects in the *syn-1* mutant animals.

Fig. 4.

The open configuration of SYN-1 does not rescue defecation defects in the *aex-1* mutant. The constitutively-open configuration of SYN-1 (see Supplementary Fig. 1C for mutational sites) was expressed in the *syn-1* or *aex-1* mutant background. Rescue activity for defecation defects was examined in each transgenic animal. SYN-1(+); *Psyn-1::syn-1*(+) genomic DNA, SYN-1(OPEN); *Pelt-2::SYN-1*(OPEN) cDNA.