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Report

UNC-13 Interaction with Syntaxin Is Required for Synaptic Transmission

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Summary

Neurotransmitter secretion at synapses is controlled by several processes—morphological docking of vesicles at release sites, priming of docked vesicles to make them fusion competent, and calcium-dependent fusion of vesicles with the plasma membrane [1, 2]. In worms, flies, and mice, mutants lacking UNC-13 have defects in vesicle priming [3–5]. Current models propose that UNC-13 primes vesicles by stabilizing Syntaxin's "open" conformation by directly interacting with its amino-terminal regulatory domain [6–8]. However, the functional significance of the UNC-13/Syntaxin interaction has not been tested directly. A truncated protein containing the Munc homology domains (MHD1 and MHD2) and the carboxy-terminal C2 domain partially rescued both the behavioral and secretion defects of *unc-13* mutants in *C. elegans*. A double mutation in MHD2 (F1000A/K1002A) disrupts the UNC-13/Syntaxin interaction. The rate of endogenous synaptic events and the amplitude of nerve-evoked excitatory postsynaptic currents (EPSCs) were both significantly reduced in UNC-13S(F1000A/K1002A). However, the pool of primed (i.e., fusion-competent) vesicles was normal. These results suggest that the UNC-13/Syntaxin interaction is conserved in *C. elegans* and that, contrary to current models, the UNC-13/Syntaxin interaction is required for nerve-evoked vesicle fusion rather than synaptic-vesicle priming. Thus, UNC-13 may regulate multiple steps of the synaptic-vesicle cycle.

Results and Discussion

Characterizing the Minimal Functional Domain of UNC-13S

Mutants lacking UNC-13 are characterized by an almost complete elimination of synaptic-vesicle fusion and by severe locomotion defects [3]. In *C. elegans*, there are two *unc-13* promoters that lead to the production of two proteins, UNC-13S and UNC-13L (S and L are for short and long) [9, 10]. We previously showed that UNC-13S expression is sufficient to rescue the behavioral defects of *unc-13* mutants [10]. UNC-13S contains two MHD (Munc homology) domains, a C1 domain (which binds phorbol esters and DAG), and two C2 domains (C2b and C2c) (Figure 1). To further map the minimal domain required for UNC-13 function, we constructed a series of truncated UNC-13S proteins and examined their ability to rescue the locomotion defects of *unc-13* mutants (Figure 1). Truncated proteins lacking MHD2 and C2c and those lacking C2c did not exhibit rescuing activity. By contrast, expressing a truncated protein corresponding to the last 900 amino acids of UNC-13S (containing MHD1, MHD2, and C2c) produced significantly improved locomotion in *unc-13* mutants.

The improved locomotion of animals expressing this truncated protein, UNC-13S(MMC), suggested that it restored a significant level of synaptic transmission. To directly assess synaptic transmission, we recorded excitatory post-synaptic currents (EPSCs) from body muscles (Figure 2). After expression of UNC-13S(MMC), the rate of endogenous vesicle fusions, measured as endogenous EPSCs in the presence of calcium, the size of the primed pool of vesicles (assayed by sucrose-evoked EPSCs), and the amplitude of nerve-evoked EPSCs were all significantly improved in comparison to those observed in *unc-13(s69)* mutants (Figure 2). The sucrose-evoked and nerve-evoked responses were rescued to 13% and 19% of wild-type levels, respectively, suggesting that UNC-13S(MMC) rescues priming and calcium-dependent exocytosis to a similar extent. UNC-13S(MMC) abundance in the ventral cord, based on expression of the GFP tag, was less than that observed for full-length UNC-13S (data not shown). A combination of decreased stability and decreased trafficking to nerve terminals probably accounts for the incomplete rescuing activity of UNC-13S(MMC). Analogous truncated forms of Munc13-1 are also sufficient to promote fusion of secretory granules in chromaffin cells (see accompanying manuscript by Stevens et al. in this issue of Current Biology [11]). We conclude from these results that the MHD1, MHD2, and C2c domains can partially reconstitute UNC-13S/Munc13-1 function and that these domains contain regions required for UNC-13 priming function.

Interestingly, UNC-13S(MMC) expression was sufficient to restore a nearly normal frequency of endogenous vesicle fusions, i.e., calcium-dependent EPSCs driven by endogenous activity, despite a decreased readily releasable pool and nerve-evoked response. These data differ from published findings that suggest a direct and proportional relationship exists between the readily releasable pool and the rate of miniature EPSCs [12, 13]. Further experiments are required to explain this potentially interesting finding.

Identifying MHD2 Residues Required for UNC-13S Interaction with Syntaxin

The UNC-13S(MMC) fragment contains Munc13-1 regions required for interactions with Syntaxin and

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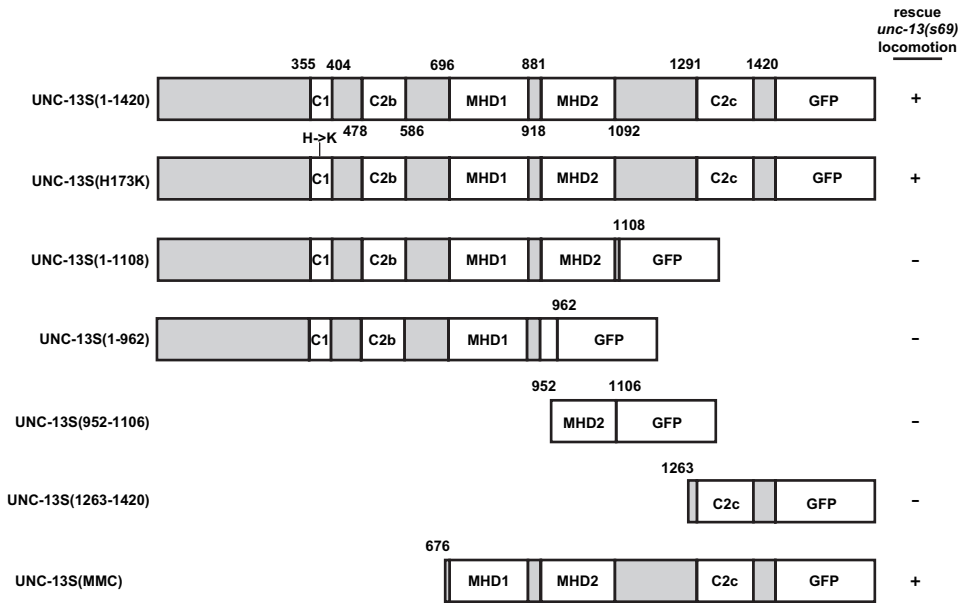

Figure 1. UNC-13S(MMC) Rescue of *unc-13* Locomotion Defects

To identify functionally important regions of UNC-13S, we expressed the depicted truncations in *unc-13(s69)* mutants. *unc-13(s69)* mutants exhibit almost no spontaneous locomotion. Transgenic animals were observed for spontaneous locomotion. + indicates rescue of *unc-13(s69)* locomotion was observed. – indicates that no observable change in *unc-13(s69)* locomotion was observed. Amino acid residues of the C1, C2, and MHD regions are indicated above and below the boxes depicting the expressed *unc-13S* regions. Transgenes expressing UNC-13S(1-1420), UNC-13S(H173K), UNC-13S(1-1108), and UNC-13S(1-962) were all expressed from the *unc-13S* internal promoter. Transgenes expressing UNC-13S(952-1106), UNC-13S(1263-1420), and UNC-13S(MMC) were expressed from the *snb-1* (synaptobrevin) promoter. All transgenes were found to be expressed throughout the nervous system, based on expression of the GFP tag. The UNC-13S(H173K) transgene was previously described [10].

GRP1/mSec7 [6, 14]. Therefore, our results were consistent with the idea that the Syntaxin and GRP1/mSec7 interactions are important for UNC-13 function. To determine if these interactions also occur with the *C. elegans* proteins, we assayed these interactions with the yeast two-hybrid system. The MHD2 domain fused to the LexA DNA binding domain positively interacted with a prey protein containing the Syntaxin Habc regulatory domain (Figure 3A). By contrast, we failed to observe interaction among full-length GRP-1, the *C. elegans* ortholog of mammalian mSec7, and several LexA-UNC-13S fusions. Fusion proteins containing the MHD2 and C2c domain weakly interacted with the Syntaxin Habc region, whereas those containing the MHD1, MHD2, and C2c domain failed to interact at all (Figure 3A). These data imply that intramolecular interactions (perhaps between MHD2 and the C2c) may occlude MHD2 binding to Syntaxin. These results indicate that the UNC-13/Syntaxin interaction is conserved in *C. elegans* but that the interaction with GRP1/mSec7 may not be (Figure 3A).

To address the functional importance of the UNC-13/Syntaxin interaction, we designed mutations in MHD2 that interfere with this interaction. Alignment of MHD2 sequences from worm, fly, rat, and mouse proteins identified a set of conserved residues (Figure 3B). Site-directed mutations that altered conserved residues to alanine were introduced. By this approach, we found that a double mutation, F1000A/K1002A, significantly reduced the MHD2/Syntaxin yeast two-hybrid interaction, whereas mutating other conserved residues had no effect on this interaction (Figure 3C). Quantitative β-galactosidase assays suggested that the F1000A/K1002A mutant MHD2 protein had <10% residual binding activity, which was not significantly different from the activity observed with the LexA negative control (Figure 3D).

The UNC-13S(F1000A/K1002A) Protein Rescues the Locomotion and Aldicarb-Resistance Phenotypes of *unc-13* Mutants

To test the importance of the UNC-13/Syntaxin interaction for synaptic transmission in vivo, we incorporated the MHD2 F1000A/K1002A double mutation into full-length UNC-13S expression constructs and expressed the resulting mutant protein in *unc-13(s69)* mutants. Expression of UNC-13S(F1000A/K1002A) or wild-type UNC-13S transgenes produced similar improvements in the locomotion behavior of *unc-13(s69)* mutants (data not shown).

To estimate the steady-state rate of acetylcholine (ACh) secretion at neuromuscular junctions (NMJs), we measured the responsiveness of transgenic animals to the cholinesterase inhibitor aldicarb (Figure S1 in the Supplemental Data available with this article online). Aldicarb treatment causes accumulation of ACh at neuromuscular junctions (NMJs), leading to acute paralysis and subsequent death. Mutations that decrease ACh secretion confer resistance to aldicarb-induced paralysis and lethality [15, 16]. Wild-type UNC-13S and UNC-13S(F1000A/K1002A) transgenes both fully rescued the aldicarb resistance of *unc-13(s69)* mutants [the fraction paralyzed after 100 min of aldicarb treatment was 96 ± 3%, wild-type UNC-13S; 90 ± 3%, UNC-13S(F1000A/K1002A); and 21 ± 6%, *unc-13(n2813)*] (Figure S1). These

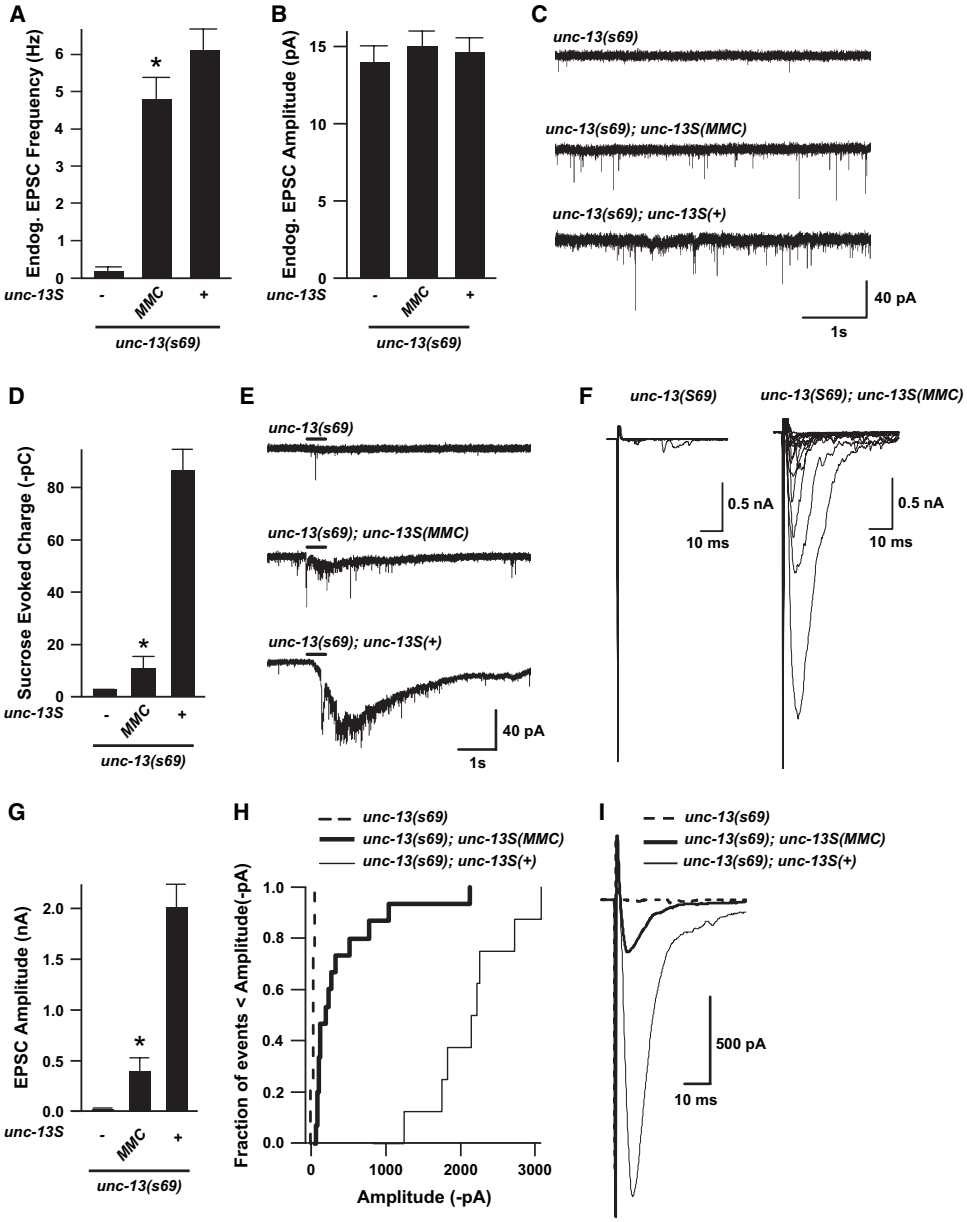

Figure 2. Electrophysiological Analysis of UNC-13S(MMC) Synaptic Function

To assess the extent of UNC-13S(MMC) rescue of *unc-13(s69)* mutants, we recorded excitatory post-synaptic currents (EPSCs) from body-wall muscle.

(A–C) Endogenous EPSCs (endog. EPSCs) were recorded in 3 mM $CaCl_2$/2mM $MgCl_2$ from body-wall muscles in adult *unc-13(s69)* animals expressing UNC-13S(MMC). The frequency (A) and amplitude (B) of endogenous EPSCs recorded in *unc-13(s69)* (n = 5), *unc-13(s69);unc-13S(+)* (n = 8), and *unc-13(s69);unc13S(MMC)* (n = 15) mutants is compared. The frequency of endogenous EPSCs was significantly ($p < .001$) increased in *unc-13(s69);unc13S(MMC)* animals compared to *unc-13(s69)*mutants. The amplitudes of endogenous EPSCs observed in *unc-13(s69);unc13S(MMC)* and *unc-13(s69);unc-13S(+)* animals were not significantly different. (C) Representative traces for each genotype compared in (A) and (B) are shown.

(D and E) Sucrose-evoked responses were recorded from body-wall muscles in adult *unc-13(s69);unc-13S(MMC)* animals. The charge transfer for each response was measured for 1 s after initiation of the stimulus. (D) Sucrose responses in *unc-13(s69)* (n = 5), *unc-13(s69);unc-13S(+)* (n = 8), and *unc-13(s69);unc13S(MMC)* (n = 8) mutants were compared. (E) Representative traces for each genotype compared in (D) are shown. In comparison to that in to *unc-13(s69)* mutants, the sucrose-evoked response in *unc-13(s69);unc-13S(MMC)* animals was significantly rescued.

(F–I) Nerve-evoked responses were recorded from body-wall muscles in adult *unc-13(s69)* (n = 5), *unc-13(s69);unc13S(+)* (n = 8), and *unc-13(s69);unc13S(MMC)* (n = 15) mutants. Ventral-cord neurons were electrically stimulated, and the post-synaptic response was recorded as described in the Supplemental Experimental Procedures. (F) Families of peak nerve-evoked responses are shown for *unc-13(s69)* and *unc-13(s69);unc13S(MMC)* animals. (G) The average peak amplitudes of the nerve-evoked responses shown in (F) are compared. (H) Cumulative probability histograms are shown for the data in (G). Compared to that in *unc-13(s69)* mutants, the nerve-evoked response in *unc-13(s69);unc-13S(MMC)* animals was significantly rescued. (I) The average nerve-evoked responses for *unc-13(s69)*, *unc-13(s69);unc13S(+)*, and *unc-13(s69);unc13S(MMC)* animals are shown. Error bars represent SEM. An asterisk indicates values that differ significantly ($p < 0.01$) from those of *unc-13(s69)* mutants.

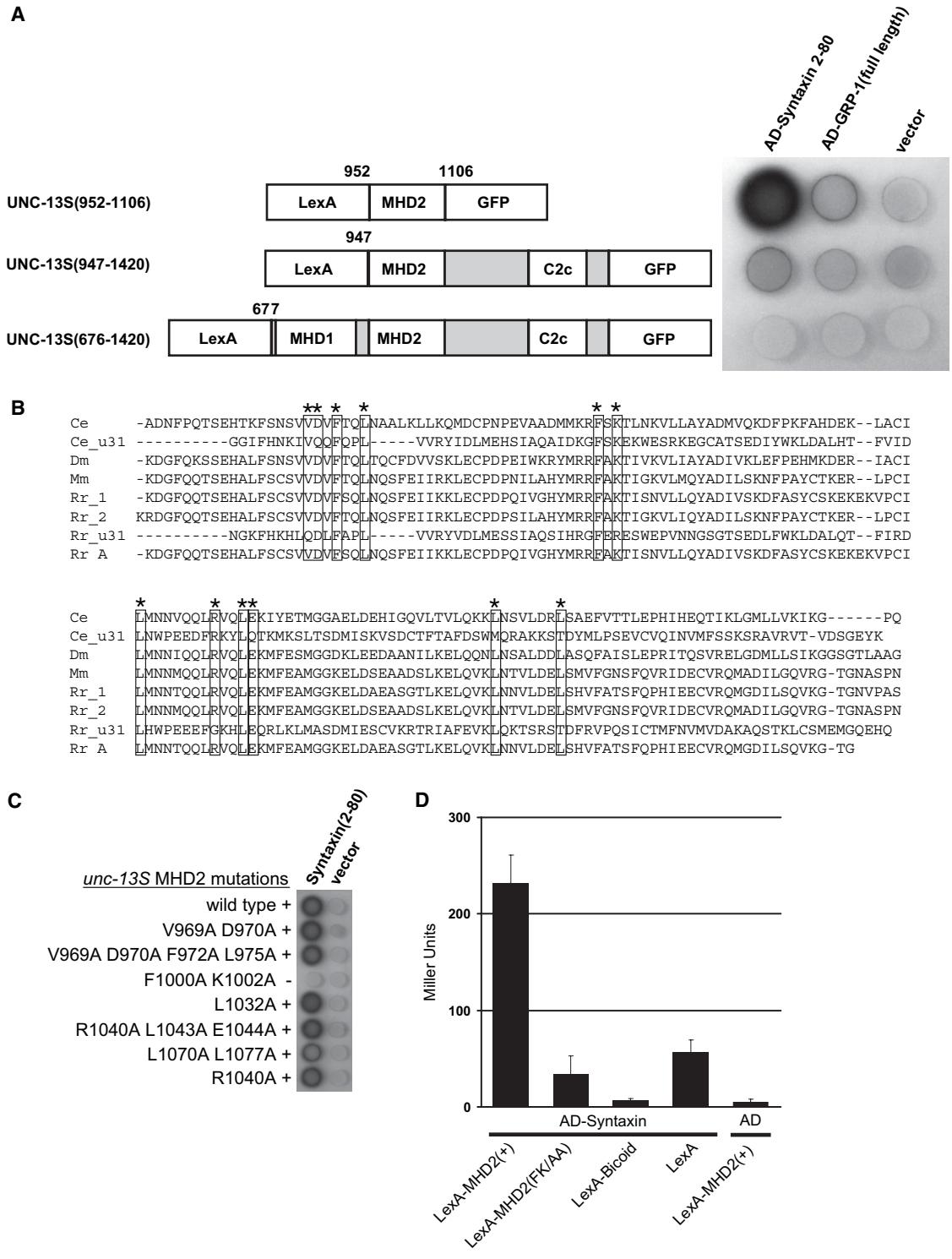

Figure 3. Identification of Mutations that Disrupt the UNC-13/Syntaxin Interaction

(A) The interaction of *C. elegans* UNC-13 with Syntaxin or GRP-1 was tested in a yeast two-hybrid assay. The *unc-13S* "bait" regions fused to LexA and GFP for each strain are indicated at the left of the figure, and the Syntaxin (amino acids 2–80) and GRP-1 (full-length) activation domain "preys" are indicated at the top. β-gal expression (dark spots) indicates interaction. Vector strains contain the vector pJG4-5 instead of the indicated bait plasmids. As judged by spot color, UNC-13S(952–1106) showed a strong interaction, UNC-13S(947–1420) showed a weak interaction, and UNC-13S(676–1420) showed no interaction. Based on expression of the GFP tag, all UNC-13 fusion proteins were judged to be expressed. A yeast two-hybrid assay was used to identify UNC-13S MHD2 mutations that disrupt the UNC-13/Syntaxin interaction.

(B) The alignment shown was used to identify a set of conserved MHD2 residues to be mutated to alanine and subsequently tested in the yeast two-hybrid assay for interaction with Syntaxin. The protein fragments used in the alignment were as follows: Ce, *C. elegans* UNC-13S(945–1134); Ce_u31, *C. elegans* UNC-31(918–1088), Dm – *D. melanogaster unc-13B* (1212–1410); Mm, *Munc13-1*(1086–1279); Rr_1, *R. norvegicus Munc-13-1*(1179–1374); Rr_2, *R. norvegicus Munc-13-2*(1461–1654); Rr_u31, *R. norvegicus* CAPS(951–1119); and Rr_A, *R. norvegicus unc-13A* (1181–1343).

results suggest that significant UNC-13 function persists after disrupting the UNC-13/Syntaxin interaction. The residual activity could reflect the residual ability of UNC-13S(F1000A/K1002A) to bind to Syntaxin. Alternatively, it is possible that the residual activity is a consequence of UNC-13 activities that are independent of its interaction with Syntaxin. Further experiments are required to distinguish between these possibilities.

The UNC-13S(F1000A/K1002A) Protein Is Defective for Synaptic Transmission

To directly assay the ability of UNC-13S(F1000A/K1002A) to promote synaptic-vesicle fusion, we recorded EPSCs in body-wall muscles (Figure 4). Compared to animals expressing wild-type UNC-13S, those expressing UNC-13S(F1000A/K1002A) had an approximately 5-fold reduction in the rate of endogenous synaptic-vesicle fusions (Figures 4A–4C) and a 40% reduction in the amplitude of nerve-evoked EPSCs (Figures 4F and 4G). Interestingly, UNC-13S(F1000A/K1002A) animals had a slight decrease (11%) in the sucrose-sensitive (i.e., primed) pool of vesicles, but this effect was not significant ($p = 0.4$) (Figures 4D and 4E). These results do not exclude the possibility that the UNC-13/Syntaxin interaction is required for vesicle priming. The F1000A/K1002A double mutant was designed based on disrupting the interaction of the isolated MHD2 and Habc domains. Consequently, the full-length UNC-13S(F1000A/K1002A) protein may have some residual affinity for Syntaxin. The UNC-13S(F1000A/K1002A) defects in the rate of endogenous EPSCs and in nerve-evoked EPSCs are both consistent with a defect in calcium-evoked vesicle fusion. The relatively normal sucrose-evoked EPSC suggests that the pool of primed vesicles is relatively normal in UNC-13S(F1000A/K1002A) mutants. Therefore, these results are most consistent with the idea that the F1000A/K1002A mutation disrupts a post-priming step in vesicle fusion.

Analogous mutations that disrupt the Munc13-1/Syntaxin interaction in mouse chromaffin cells significantly decreased fusion of secretory granules (see accompanying manuscript, Stevens et al. [11]). Thus, the UNC-13/Syntaxin interaction is conserved across phylogeny and is required for UNC-13 function in the calcium-dependent secretion of both synaptic vesicles and secretory granules. Interestingly, in the Munc13-1 case, the secretion defects were apparently caused by decreased priming of secretory granules. This difference could reflect differences in the preparations, or perhaps different roles for UNC-13 proteins in the secretion of synaptic vesicles versus secretory granules. Thus, although both studies support the functional importance of the UNC-13/Syntaxin interaction, precise conclusions concerning why priming is differentially impacted in the two systems will require further studies.

One possible explanation for our results is that the UNC-13S(F1000A/K1002A) mutation decreases calcium entry or the calcium sensitivity of synaptic-vesicle fusion. For example, UNC-13 might regulate the interaction between Syntaxin and presynaptic calcium channels. Prior studies have shown that Syntaxin inhibits calcium influx through voltage-gated calcium channels [17] and that this interaction can regulate transmission at *Drosophila* NMJs [18]. If this were the case, we would expect that the UNC-13S(F1000A/K1002A) secretion defect would be corrected by an increase in extracellular calcium concentration. However, the UNC-13S(F1000A/K1002A) mutation still caused a significant defect in both the rate of endogenous EPSCs and the amplitude of nerve-evoked EPSCs in high-calcium saline solutions (Figures 4A, 4C, 4F, and 4G). In fact, the amplitude of nerve-evoked EPSCs (for both wild-type and mutant animals) was not significantly different in the high-calcium solution, suggesting that under these conditions calcium levels are saturating. Therefore, these results suggest that the UNC-13S(F1000A/K1002A) secretion defect is still observed under conditions where calcium is saturating. These results suggest that UNC-13S(F1000A/K1002A) causes a defect in a calcium-independent step of secretion that occurs after vesicle priming.

These results have several implications for previous models of the synaptic-vesicle cycle and UNC-13′s role in priming and synaptic-vesicle fusion. The pool of primed and docked vesicles can be assayed as the readily releasable pool (RRP) of vesicles [13]. The size of the RRP varies significantly among synapses and can be regulated by presynaptic metabotropic signaling pathways [19, 20]. Thus, there is significant interest in determining the biochemical mechanisms that define and regulate vesicle priming. Current data support a model whereby UNC-13 promotes vesicle priming via stabilizing Syntaxin's "open" conformation, which is competent to form SNARE complexes and mediate vesicle fusions. This model is supported by two critical experiments. First, Munc13-1 [6] and UNC-13 (Figure 3) both bind to the amino-terminal inhibitory domain (Habc) of Syntaxin. Thus, it seems likely that formation of the UNC-13/Syntaxin complex would destabilize the closed conformation in which Habc forms an intramolecular contact with its SNARE helix [7]. Second, expression of a Syntaxin mutant form that is constitutively in the open conformation reconstitutes synaptic-vesicle fusion in *unc-13* mutants [8]. Our results, and those in the accompanying manuscript by Stevens et al. [11], provide further support for this model by showing that the UNC-13/Syntaxin interaction is conserved in *C. elegans* and that this interaction is required for reconstituting the full synaptic vesicle fusion activity of UNC-13. The MHD2 residues implicated in Syntaxin binding (F1000/K1002) are conserved in worm, fly, and

(C) A reverse two-hybrid assay identifies the F1000A/K1002A mutations that disrupt the UNC-13/Syntaxin interaction. An X-gal overlay assay was performed on yeast strains expressing either wild-type or the mutated LexA-UNC-13(MHD2)-GFP bait indicated at the left, in combination with either a Syntaxin(2–80) prey plasmid or the vector (pJG4-5). The F1000A/K1002A UNC-13MHD2 (pQT#168) plasmid was the only mutant found to disrupt the UNC-13/Syntaxin interaction. All strains carrying wild-type or mutant LexA-UNC-13-MHD2 plasmids expressed GFP.
(D) The wild-type and MHD2(F1000A/K1002A) mutant two-hybrid interactions were compared by quantitative liquid β-gal assay. The yeast strains with the bait plasmids LexA-UNC13S-MHD2-GFP (LexA-MHD2(+)) and the LexA-UNC13S-MHD2(F1000A/K1002A)-GFP (LexA-MHD2(F1000A/K1002A)), LexA-Bicoid (pRFHM1), and vector (pJG4-5) alone were expressed with either the Syntaxin(2–80) prey plasmid (KP#1327) (AD-Syntaxin) or vector alone (AD). The β-gal activity is expressed in Miller Units. Error bars represent SEM.

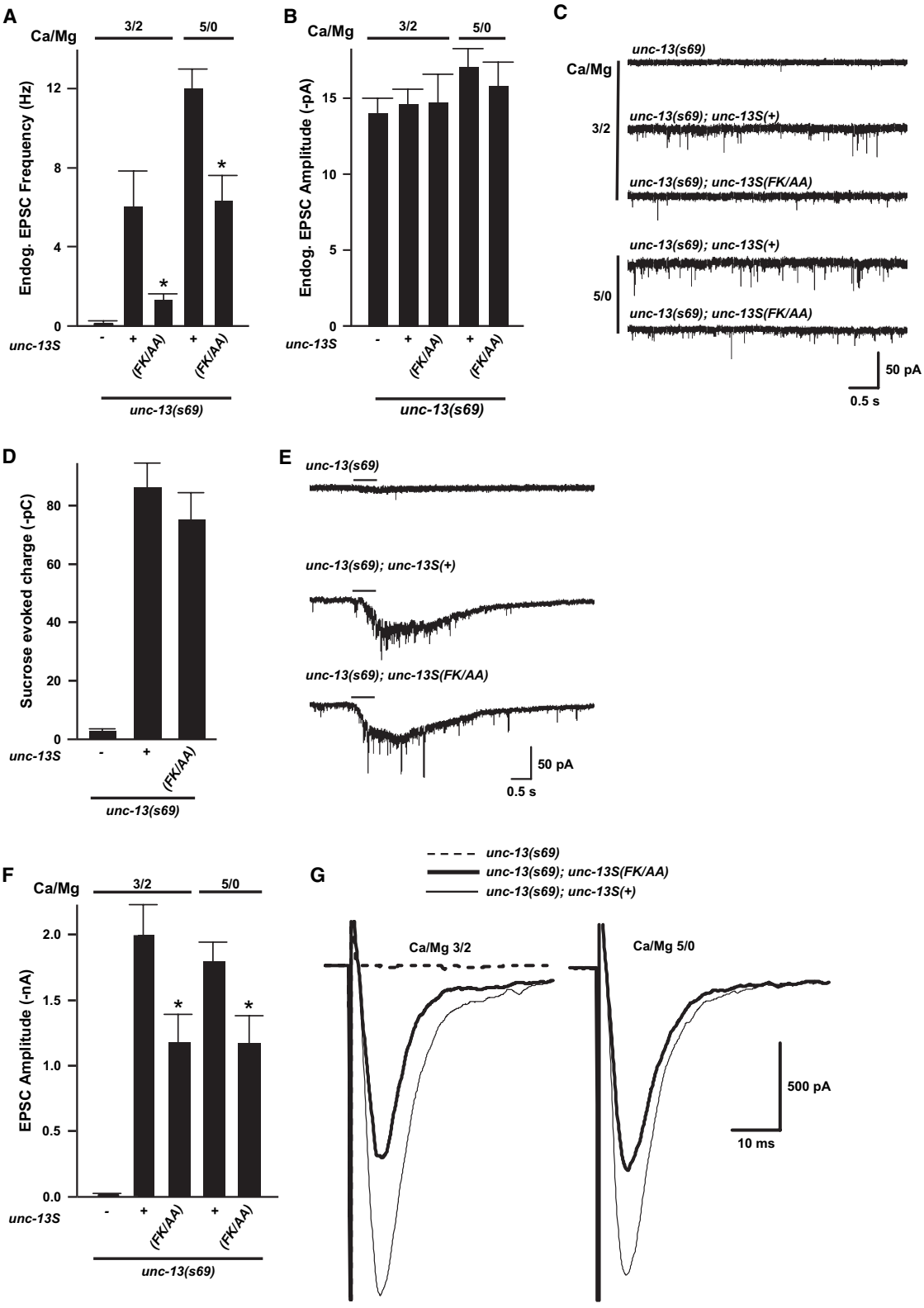

Figure 4. *unc-13S(F1000A/K1002A)* Mutants Have a Defect in Nerve-Evoked Vesicle Fusion

To assess the synaptic function of UNC-13S(F1000A/K1002A), we performed whole-cell recordings from body-wall muscle and recorded postsynaptic currents in *unc-13(s69)*, *unc-13(s69);unc-13S(+)*, and *unc-13(s69);unc13S(F1000A/K1002A)* animals.

(A–C) Endogenous EPSCs were recorded in 3 mM $CaCl_2$/2 mM $MgCl_2$ from body-wall muscles in adult *unc-13(s69) unc-13S(F1000A/K1002A)* animals in normal calcium (3 mM Ca/2 mM Mg) or high (5 mM Ca/0 mM Mg) calcium. The frequency (A) and amplitude (B) of endogenous EPSCs recorded in *unc-13(s69)* (n = 5), *unc-13(s69);unc-13S(+)* (n = 8), and *unc-13(s69);unc13S(F1000A/K1002A)* (n = 8) mutants in normal and high calcium are compared. A significant decrease in the frequency of endogenous EPSCs was observed in *unc-13(s69);unc13S(F1000A/K1002A)*

vertebrate UNC-13 proteins, suggesting that the interaction with Syntaxin is also conserved across phylogeny. Electrophysiological recordings show that UNC-13S (F1000A/K1002A) mutants had significantly reduced synaptic vesicle fusions, both endogenous and nerve-evoked. Thus, impairing the UNC-13/Syntaxin interaction results in defective synaptic transmission.

A surprising aspect of our work was that the UNC-13S(F1000A/K1002A) secretion defect was due to a defect in a calcium-independent step of nerve-evoked vesicle fusion. Contrary to expectation, we found that the pool of sucrose-responsive vesicles was not significantly reduced in UNC-13S(F1000A/K1002A) mutants. Although these results validate the functional importance of the UNC-13/Syntaxin interaction, they imply that UNC-13's role in synaptic transmission is not limited to vesicle priming, as defined by sucrose-evoked vesicle fusion. Our results suggest that UNC-13 plays several roles in making vesicles fusion competent. UNC-13 is required for the formation of a sucrose-sensitive pool of vesicles (sucrose-primed), whereas interactions between UNC-13 and Syntaxin promote a pool of vesicles that are now responsive to calcium (calcium primed). Therefore, these results suggest a broader view of vesicle priming whereby multiple interactions between SNAREs and regulatory proteins promote maturation of vesicles to a fully fusion-competent state.

Supplemental Data

Supplemental Data are available with this article online at http://www.current-biology.com/cgi/content/full/15/24/2236/DC1/.

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animals compared to *unc-13(s69);unc-13S(+)* controls. The amplitude of endogenous EPSCs observed in *unc-13(s69);unc13S(F1000A/K1002A)* and *unc-13(s69);unc13S(+)* animals were not significantly different. (C) Representative traces from each genotype shown in (A) and (B) are shown. (D and E) Sucrose-evoked responses were recorded from body-wall muscles in adult *unc-13(s69);unc-13S(F1000A/K1002A)* animals. The charge transfer for each response was measured for 1 s after the initiation of the stimulus. (D) Sucrose responses in *unc-13(s69)* (n = 5), *unc-13(s69);unc-13S(+)* (n = 5), and *unc-13(s69);unc13S(F1000A/K1002A)* (n = 5) mutants were compared. (E) Representative traces from each genotype in (D) are shown. Compared to that in *unc-13(s69); unc-13S(+)* animals, the sucrose-evoked response in *unc-13(s69); unc-13S(F1000A/K1002A)* animals was not significantly altered.

(F and G) Nerve-evoked responses were recorded from body-wall muscles in adult *unc-13(s69)* (n = 5), *unc-13(s69)*; *unc13S(+)* (n = 8), and *unc-13(s69);unc13S(F1000A/K1002A)* (n = 8) mutants. Ventral-cord neurons were electrically stimulated, and the post-synaptic response was recorded. (F) The average peak amplitudes of the nerve-evoked responses for *unc-13(s69)*, *unc-13(s69);unc13S(+)*, and *unc-13(s69);unc13S(F1000A/K1002A)* animals are compared. The nerve-evoked response in *unc-13(s69);unc-13S(F1000A/K1002A)* showed a significant decrease in comparison to that in *unc-13(s69);unc13S(+)* animals. (G) The average nerve-evoked responses for *unc-13(s69)*, *unc-13(s69);unc13S(+)*, and *unc-13(s69);unc13S(F1000A/K1002A)* animals are shown. Error bars represent SEM. An asterisk indicates values that differ significantly ($p < 0.01$) from those of *unc-13(s69);unc13S(+)* controls.