UNC-13 is required for synaptic vesicle fusion in *C. elegans*

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We analyzed the synaptic physiology of *unc-13* mutants in the nematode *C. elegans*. Mutants of *unc-13* had normal nervous system architecture, and the densities of synapses and postsynaptic receptors were normal at the neuromuscular junction. However, the number of synaptic vesicles at neuromuscular junctions was two- to threefold greater in *unc-13* mutants than in wild-type animals. Most importantly, evoked release at both GABAergic and cholinergic synapses was almost absent in *unc-13* null alleles, as determined by whole-cell, voltage-clamp techniques. Although mutant synapses had morphologically docked vesicles, these vesicles were not competent for release as assayed by spontaneous release in calcium-free solution or by the application of hyperosmotic saline. These experiments support models in which UNC-13 mediates either fusion of vesicles during exocytosis or priming of vesicles for fusion.

Mutants of *unc-13* were originally identified by Sydney Brenner based on their severely uncoordinated phenotype[1]. Strong alleles such as *unc-13(s69)* are almost completely paralyzed[2]. Further characterization of the phenotype suggested that *unc-13* mutants have a presynaptic defect in neurotransmission. First, *unc-13* mutants are resistant to inhibitors of acetylcholinesterase, which indicates that there are decreased levels of acetylcholine in the synaptic clefts of mutants[3,4]. Nevertheless, these mutants accumulate high levels of acetylcholine in their tissues[3,4], indicating that the neurons in these mutants are defective in the release of acetylcholine. Finally, these mutants are sensitive to the acetylcholine agonist levamisole, which demonstrates that acetylcholine receptors are present and functional on the postsynaptic membrane of the muscle[3]. Together, these data suggest that the paralyzed phenotype of *unc-13* mutants is caused not by defects in the postsynaptic cells but rather by a presynaptic defect in the release of neurotransmitter.

The UNC-13 protein is a large protein with multiple isoforms, which contain two or three C2 motifs and one C1 motif[5]. Some C2 domains bind phospholipids and calcium[6]. C1 domains can bind diacylglycerol and serve both to anchor a protein to the plasma membrane and to activate it[7]. Two major isoforms, which differ by the presence of alternative amino termini (**Fig. 1**) are encoded by *unc-13*. These isoforms are named LR and MR, according to their domain structures (R. Kohn and J. Rand, personal communication). The structure of UNC-13 proteins is conserved in *Drosophila* and vertebrates. Specifically, vertebrates have three *Munc13* genes[8], all of which encode proteins with the conserved R domain[9]. In addition, *Munc13-1* encodes a protein containing a divergent amino terminal C2 domain just like the LR transcript of UNC-13. In *Drosophila*, a single gene encodes three forms, all of which contain the highly conserved R domain but none of which contain the amino terminal C2 domain[10,11]. The biochemical activities of the UNC-13 C2 domains have not yet been analyzed, but three lines of evidence suggest that the C1 domain of these proteins is likely to mediate diacylglycerol signaling, as shown for the C1 domains of PKC. First, both UNC-13 and Munc13 bind phorbol esters[5,12–14]. Second, phorbol ester binding causes Munc13-1 to translocate to the plasma membrane[12]. Third, accumulation of diacylglycerol caused by mutations in diacylglycerol kinase potentiate synaptic transmission, probably by an UNC-13-mediated mechanism[15].

To determine the role of UNC-13 at the synapse, we characterized the physiology of the neuromuscular junction in an allelic series of *unc-13* mutations. The *n2813* mutants are only weakly uncoordinated. The *e1091* and *e51* mutants, with null alleles of the LR isoform[5], are strongly uncoordinated. The *s69* mutants, with a null allele for both the LR and MR isoforms (R. Kohn and J. Rand, personal communication), are severely uncoordinated. These mutations affect neurotransmission in a progressively more severe manner, and the null allele virtually eliminates the release of synaptic vesicles. Our data support a role for UNC-13 after docking, either in vesicle fusion or in the formation of fusion-competent vesicles at the active zone of synapses.

Results

Mutations of *unc-13* result in severe uncoordination and defects in neurotransmission. One possible explanation for these defects is abnormal wiring of the motor neurons. To examine the morphology of GABAergic motor neurons, we analyzed the distribution of green fluorescent protein (GFP) expressed under the *unc-47* promoter in the wild type and in *unc-13* mutants. The *unc-47* gene encodes the vesicular GABA transporter and is expressed only in the GABAergic neurons of *C. elegans*[16]. In *unc-13(e1091)* and *unc-13(s69)* mutants, GABAergic motor neurons extend processes to both ventral and dorsal muscle quadrants, and overall the architecture of the GABAergic nervous system is indistinguishable from that of the wild type (**Fig. 2**). Thus, the uncoordinated phenotype of *unc-13* mutants is not caused by failure of motor neurons to extend processes to muscles.

Alternatively, formation of either pre- or postsynaptic elements of the neuromuscular junction might be disrupted. We determined whether neuromuscular junctions were present by visualizing clusters of synaptic vesicles tagged with GFP

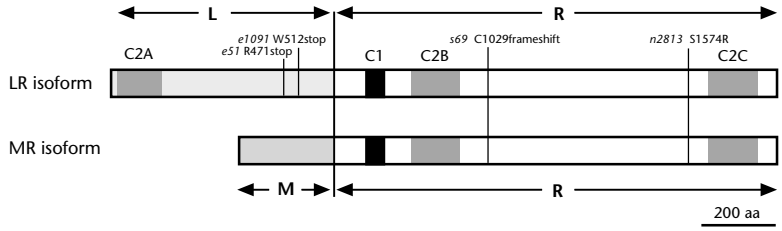

Fig 1. Domain structure of UNC-13, indicating the location of the mutations used in this study. The *e1091* and *e51* mutations are stop codons in the L region and thus represent null alleles of the LR isoform[5]. The *s69* mutation is a five-basepair deletion in the R region and causes a frameshift in the common domain of the protein isoforms (Kohn and Rand, personal communication). The region between C2B and C2C is highly conserved among *C. elegans, Drosophila* and mammalian UNC-13 proteins; because *s69* eliminates this region, this allele is likely to be a null mutation in both the LR and MR isoforms. The *n2813* mutation is a missense change in the common R domain and thus represents a hypomorphic allele that affects all isoforms.

(**Fig. 3a–c**). Specifically, we assayed the distribution of GFP-tagged synaptobrevin expressed in the GABAergic neurons under the control of the *unc-25* promoter[17]. Glutamic acid decarboxylase, the biosynthetic enzyme for GABA, which is only expressed in GABAergic neurons[18], is encoded by *unc-25*. The average interval between synapses in *unc-13(e1091)* mutants and *unc-13(s69)* mutants was similar to that of the wild type (**Fig. 3g**). Additionally, the postsynaptic organization of neurotransmitter receptors was normal in *unc-13* mutants. The distribution of GFP-tagged GABA receptors in *unc-13(s69)* and *unc-13(e1091)* was indistinguishable from that of the wild type (**Fig. 3d–f**). The functionality of these receptors was confirmed by pressure ejecting GABA directly onto the body wall muscles. There was no significant difference in the amplitude of the muscle response to GABA or acetylcholine application in the *unc-13* mutants when compared to the wild type (**Fig 3h** and **i**). Together these data indicate that the uncoordinated phenotype in *unc-13* mutants is not caused by defects in either synapse formation or receptor expression but is more likely the result of a functional defect of the neuromuscular junction.

Abnormal synaptic function could be caused by a defect in either synaptic vesicle endocytosis or exocytosis. A block in synaptic vesicle endocytosis would lead to depletion of synaptic vesicles, whereas a defect in exocytosis would lead to an accumulation of synaptic vesicles. Electron micrographs showed that more synaptic vesicles were present at the neuromuscular junction in *unc-13* mutants than in the wild type ($p < 0.0001$; **Fig. 4**). The number of synaptic vesicles associated with a presynaptic specialization was 3.3-fold greater in *unc-13(e1091)* and 2.5-fold greater in *unc-13(s69)* than in wild-type animals (**Fig. 4d**). Abnormal vesicle accumulation was observed in these mutants at both GABAergic synapses (wild type, 275 ± 45 synaptic vesicles per synapse; *e1091*, 1512 ± 390; *s69*, 1395 ± 247) and cholinergic synapses (wild type, 313 ± 42; *e1091*, 780 ± 162; *s69*, 545 ± 84). These data do not suggest a defect in endocytosis or in vesicle biogenesis because vesicles are plentiful and are clustered at release sites. Instead, the observed increase in synaptic vesicles suggests a defect in exocytosis in *unc-13* mutants.

A defect in neurotransmission was confirmed by directly assaying postsynaptic currents in response to nerve stimulation. We stimulated the ventral nerve cord while recording from muscles by whole-cell patch clamp. Stimulation of the ventral nerve cord in wild-type animals elicited a robust postsynaptic current (**Fig. 5**). These responses were calcium dependent, as the evoked current was reversibly abolished by removal of extracellular calcium (**Fig. 5a**). Evoked responses were greatly reduced in *unc-13* mutants compared to the wild type; specifically, current amplitude was 32% of the wild-type response in *unc-13(n2813)* ($n = 9$), 4% in *unc-13(e51)* ($n = 8$), 6% in *unc-13(e1091)* ($n = 7$) and 0% in *unc-13(s69)* ($n = 4$).

Similarly, there was a severe decrease in endogenous currents in *unc-13* mutants compared to the wild type. In 5 mM calcium, the frequency of endogenous synaptic events was robust in wild-type animals (40.0 ± 6 events per s, $n = 13$) and almost absent in the null mutant (0.14 ± 4 events per s, $n = 8$; **Fig. 6a** and **b**). Furthermore, endogenous synaptic activity was decreased at both cholinergic and GABAergic synapses in the *unc-13* mutants. Cholinergic responses were specifically blocked by D-tubocurare (0.5 mM)[19] in the wild type. The frequency of the remaining GABA-mediated events was 19 ± 4 per second ($n = 10$); thus, roughly half of the spontaneous currents are attributable to GABA.

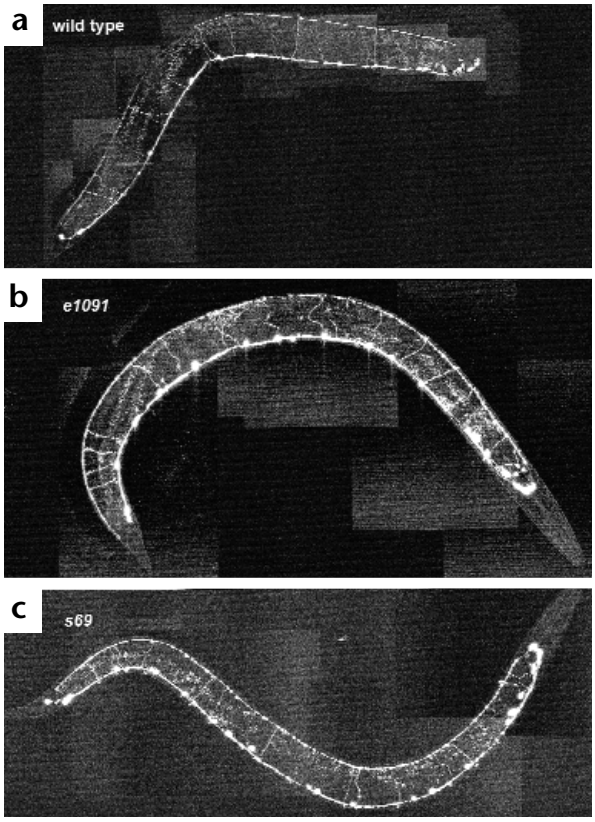

Fig 2. GABAergic motor neuron anatomy is normal in *unc-13* mutants. The *oxIs12* integrated array was used to label GABAergic neurons in the wild type (**a**), *unc-13(e1091)* (**b**) and *unc-13(s69)* (**c**). The *oxIs12* array includes a construct with green fluorescent protein (GFP) expressed under the control of the *unc-47* promoter, which is expressed in all GABAergic neurons[16]. For all figures, anterior is to the right, dorsal is up.

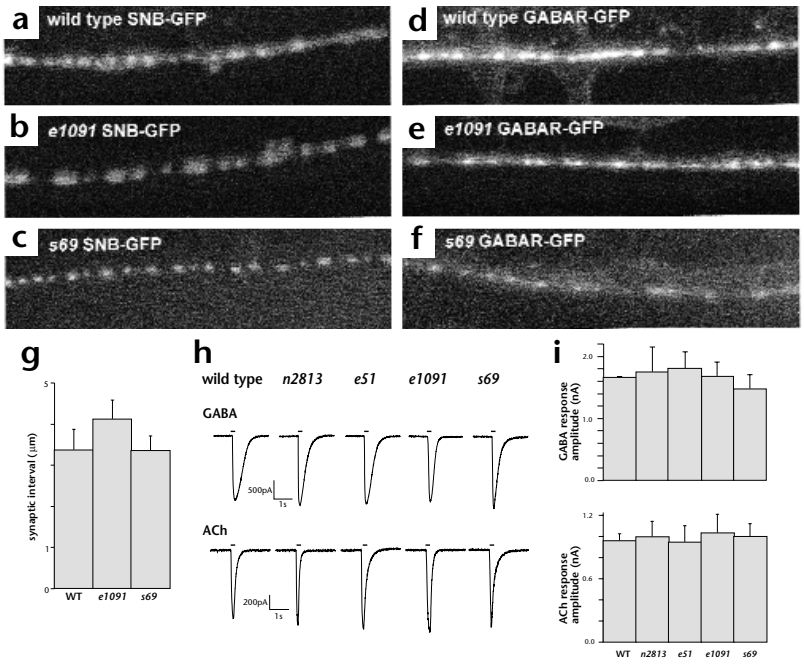

Fig. 3. The density and distribution of neuromuscular synapses is normal in *unc-13* mutants. (**a–c**) GFP-tagged synaptobrevin (SNB-GFP) localization. The integrated array *nIs52* was used to label GABAergic neuromuscular junctions in the wild type (**a**), *unc-13(e1091)* (**b**) and *unc-13(s69)* (**c**). The *nIs52* array includes a construct with GFP fused to the lumenal terminus of the synaptic vesicle protein synaptobrevin, under the regulation of the *unc-25* promoter, which is expressed in GABAergic neurons[17]. (**d–f**) Localization of postsynaptic GABA receptors (GABAR-GFP). The integrated array *oxIs22* was used to visualize GABA receptors in the wild type (**d**), unc-13(e1091) (**e**) and *unc-13(s69)* (**f**). The *oxIs22* array contains the *unc-49* locus with GFP inserted into the intracellular loop of the UNC-49B GABA receptor subunit[33]. (**g**) Interval between synapses (µm), measured as distance between SNB-GFP clusters. (**h**) Sample currents evoked by pressure ejection of GABA or acetylcholine (0.1 mM, 100 ms) onto body wall muscles of wild-type animals or *unc-13* mutants. (**i**) Average current amplitudes observed in response to GABA and acetylcholine application.

Because activity in the strong *unc-13* alleles, *e1091* and *s69*, was reduced 10-fold and 300-fold, respectively from the wild-type rate, these mutations must affect both GABAergic and cholinergic transmission, as concluded from the ultrastructural data.

The observed defect in neurotransmission might be caused by reduced sensitivity of postsynaptic receptors to neurotransmitter. Alternatively, the decrease in neurotransmission could be caused by failure of synaptic vesicles to be loaded with neurotransmitter. However, the average amplitude of spontaneous events was the same in all genotypes (**Fig. 6c**). Thus both the response of postsynaptic receptors and the transport of neurotransmitters into synaptic vesicles are normal in *unc-13* mutants.

Given that synaptic vesicles are readily available and filled with neurotransmitter, the defect in *unc-13* mutants could lie in the steps leading to exocytosis. The first step in synaptic vesicle exocytosis is docking of vesicles at the plasma membrane as defined by morphological criteria. We quantified the number of synaptic vesicles that were in contact with the plasma membrane at neuromuscular junctions and found a substantially increased number of morphologically docked vesicles in *unc-13* mutants (**Fig. 4e**). However, this increase in the absolute number of docked vesicles in mutants probably reflects the increase in total number of vesicles at each synapse, as the percentage of vesicles docked at a neuromuscular junction was similar in wild-type animals and *unc-13* mutants (**Fig. 4f**). These data suggest that the docked pool is in equilibrium with the reserve pool of vesicles, as demonstrated in *Drosophila*[20]. We conclude that the presence of docked vesicles in *unc-13* mutants indicate that the defect in exocytosis occurs after docking of vesicles to the membrane.

A defect in evoked release of docked synaptic vesicles could be caused either by a lack of fusion-competent vesicles or by a defect in calcium signaling following the stimulus. These two possibilities can be distinguished by analyzing spontaneous miniature postsynaptic currents (mPSCs), which are calcium independent. If vesicles are fusion competent (primed), then spontaneous mPSCs should still occur at roughly normal frequencies even without calcium. If vesicles are not primed, then *unc-13* mutations should reduce the spontaneous release of synaptic vesicles. In the wild type, calcium-independent mPSCs occurred at a frequency of 5.0 events per second; mPSCs were reduced in all *unc-13* mutants and were almost absent in the null mutant (0.03 events per second). Event amplitudes for the *unc-13*

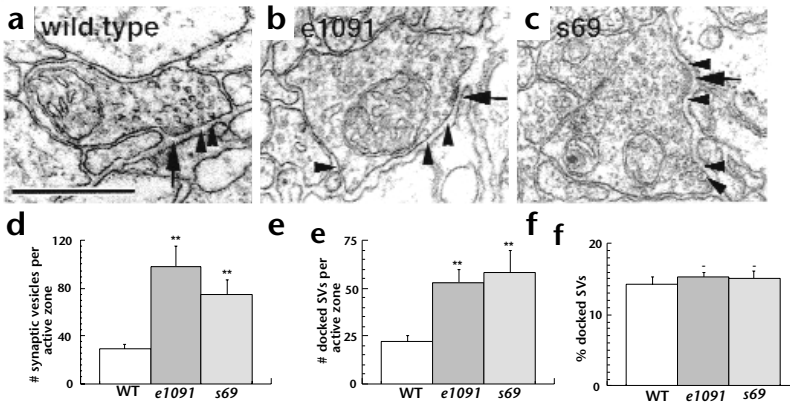

Fig. 4. Synaptic vesicles accumulate in *unc-13* mutants. Electron micrograph of GABAergic synapses in the wild type (**a**), *unc-13(e1091)* (**b**) and *unc-13(s69)* (**c**). In all micrographs, arrows indicate presynaptic specialization, and arrowheads indicate docked vesicles. (**d**) Average number of synaptic vesicles per active zone. These data include both GABAergic and cholinergic neuromuscular junctions. (**e**) Average number of docked vesicles per active zone. (**f**) Percent of vesicles docked at active zones. $^{**}p < 0.001$, $^{***}p < 0.0001$ (significantly different from wild type) - difference from wild type not statistically significant.

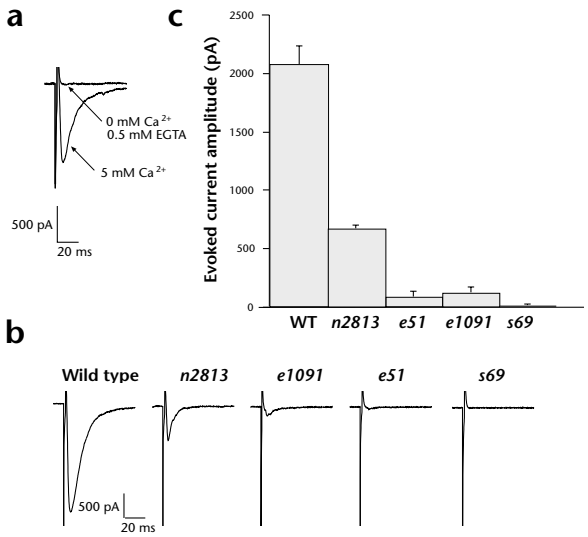

Fig. 5. Evoked responses are reduced in *unc-13* mutants. (**a**) Sample traces of evoked release in 5 mM Ca^{2+} and in 0 mM Ca^{2+} with 0.5 mM EGTA. (**b**) Sample traces of evoked responses in the muscles of the wild type, *unc-13(n2813)*, *unc-13(e51)*, *unc-13(e1091)* and *unc-13(s69)*. (**c**) Average amplitudes of evoked currents in wild-type and *unc-13* muscles.

mutants were similar to the wild-type amplitudes, supporting the conclusion that postsynaptic receptor sensitivity is not reduced at *unc-13* neuromuscular synapses. The lack of calcium-independent mPSCs indicates that the docked vesicles in *unc-13* mutants are not fusion competent.

Primed vesicles can be induced to fuse with the plasma membrane in the absence of calcium influx by application of hyperosmotic media[21–23], and the SNARE complex is required for vesicle fusion induced in this way[11]. To determine whether the docked vesicles we observed in electron micrographs were fusion competent, we applied hyperosmotic media to *unc-13* mutants. In the null allele *s69*, almost no synaptic vesicles were released in response to hyperosmotic media (**Fig. 8**). Specifically, the number of quanta released during hyperosmotic stimulation in the *unc-13* null allele *s69* was reduced by 97% relative to the wild-type response. These data demonstrate that very few morphologically docked vesicles are primed for fusion.

Discussion

Our data demonstrate that UNC-13 is required for synaptic vesicle exocytosis. Mutants of *unc-13* appear to possess a structurally normal nervous system, including a normal density of neuromuscular junctions and normal expression and localization of postsynaptic receptors in the muscle. Although these synapses contain abundant synaptic vesicles, depolarization of motor neurons largely fails to elicit vesicle release. Ultrastructural analysis demonstrated that vesicles dock at the plasma membrane near active zones. However, these docked vesicles did not spontaneously fuse with the plasma membrane in calcium-free solution nor could they be released by the application of hyperosmotic media. Similar results are obtained in the *Drosophila* UNC-13 mutant[11] and in the mouse Munc13-1 mutant[24]. However, in contrast to the Munc13-1 mutant, GABAergic synapses are defective in the *C. elegans unc-13* mutants. These data indicate that UNC-13 is required at a step after docking, either in priming vesicles for fusion or in the fusion step itself.

UNC-13 proteins are implicated in synaptic plasticity. Overexpression of Munc13-1 enhances spontaneous release of neurotransmitter at *Xenopus* neuromuscular junctions, and this increase in release is augmented by phorbol ester[12]. Similarly, $G\alpha_o$ mutants in *C. elegans* release excessive acetylcholine; in these mutants, the UNC-13 MR subunit is localized to synapses, and this localization requires the C1 phorbol ester binding domain[15]. These data suggest that UNC-13 may have a modulatory role at the synapse. Our data indicate that UNC-13 function cannot be limited to modulation of synaptic transmission, but that it is essential for the basic mechanisms of exocytosis. However, it may be possible to reconcile an essential role for UNC-13 in fusion competence with an additional role in synaptic plasticity. For example, increased levels of UNC-13 at the active zone could lead to an increase in the number of primed vesicles so that more vesicles are released by motor neuron depolarization. There are multiple isoforms of UNC-13 proteins. LR isoforms are localized at the active zone (Kohn and Rand, personal communication); MR isoforms are cytoplasmic but can be relocalized to the plasma membrane in the absence of $G\alpha_o$[15]. The LR isoforms may be responsible for essential priming functions of UNC-13 and the MR isoforms may be involved in modulatory functions of UNC-13.

Could UNC-13 be involved in calcium signaling? The LR form of UNC-13 has three C2 domains, and the MR form has two C2 domains. C2 domains are calcium-binding motifs found in a number of synaptic proteins, including synaptotagmin, DOC2 and rabphilin[6]. The first and third C2 domains of UNC-13 lack the critical aspartates that chelate calcium ions, so only the second C2 domain resembles a fully functional calcium-binding C2 domain. The presence of this C2 domain suggests that UNC-13 could be a calcium sensor, perhaps even mediating calcium-stimulated fusion of synaptic vesicles. However, because there are almost no fusion-competent vesicles in *unc-13* mutants, we cannot determine whether these mutants have additional defects in calcium sensing.

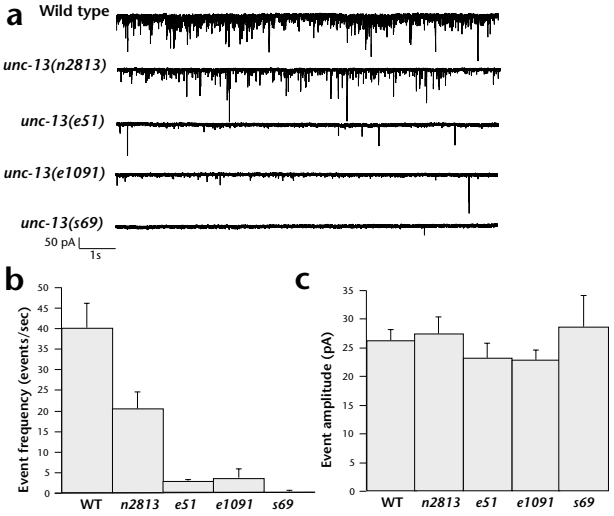

Fig. 6. Endogenous release of synaptic vesicles is reduced in *unc-13* mutants. (**a**) Sample traces of spontaneous currents in the muscles of the wild type, *unc-13(n2813)*, *unc-13(e51)*, *unc-13(e1091)* and *unc-13(s69)*. (**b**) Average frequencies of spontaneous currents in wild-type and *unc-13* muscles. (**c**) Average amplitudes of spontaneous events in wild-type and *unc-13* muscles.

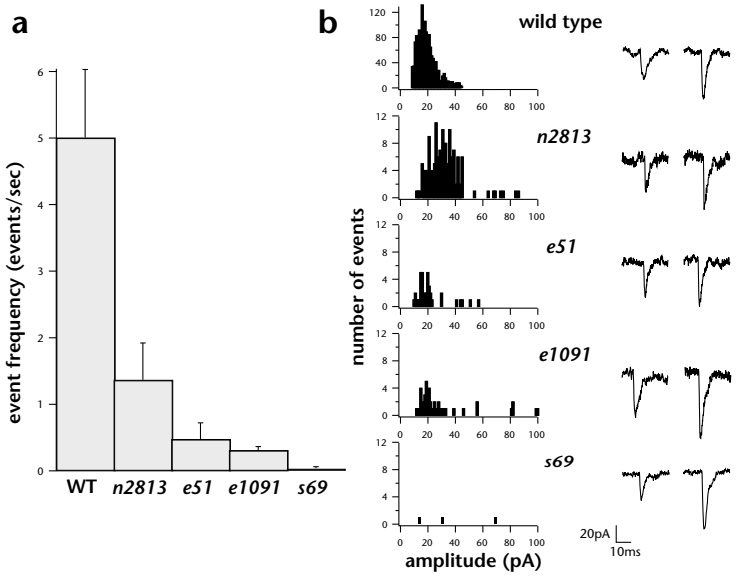

Fig. 7. Calcium-free miniature postsynaptic events are severely reduced in *unc-13* mutants. (**a**) Average frequency of calcium-free miniature postsynaptic currents in wild type (n = 7) and *unc-13* muscles (*n2813*, n = 5; *e51*, n = 5; *e1091*, n = 5; *s69*, n = 3). (**b**) Left, amplitude histograms of calcium-free miniature currents in wild-type animals and *unc-13* mutants. Right, sample traces.

How might UNC-13 function? The severe block in exocytosis observed in *unc-13* mutants is consistent with the protein being directly involved in fusion of synaptic vesicles with the plasma membrane. However, the rare fusions induced by hyperosmotic solution indicate that vesicles are not completely refractory to fusion. On the other hand, biochemically defined interactions of synaptic proteins suggest that UNC-13 could be involved in the priming of vesicles for fusion rather than the fusion event itself; specifically, UNC-13 might regulate UNC-18 interactions with syntaxin. UNC-18 binds syntaxin in vertebrates[25] and in *C. elegans*[26] and blocks the formation of the SNARE complex *in vitro*[27]. Overexpression of UNC-18 in *Drosophila* decreases the number of vesicles in the readily releasable pool[28], and mutations in syntaxin that decrease UNC-18 binding increase vesicle number in the readily releasable pool[29]. These data indicate that UNC-18 blocks the formation of the SNARE complex *in vivo*. Munc13-1 also binds syntaxin[30], and *C. elegans* UNC-13 binds UNC-18 (ref. 31). Furthermore, UNC-13 is able to displace UNC-18 from syntaxin *in vitro*[31]. The simplest model that is consistent with these biochemically identified interactions is that UNC-13 participates in the conversion of docked vesicles to primed vesicles. Specifically, UNC-13 could displace UNC-18 from syntaxin so that syntaxin could interact with the other components of the SNARE complex (that is, the plasma membrane protein SNAP25 and the vesicle-associated protein synaptobrevin[32]) and thereby generate a primed vesicle.

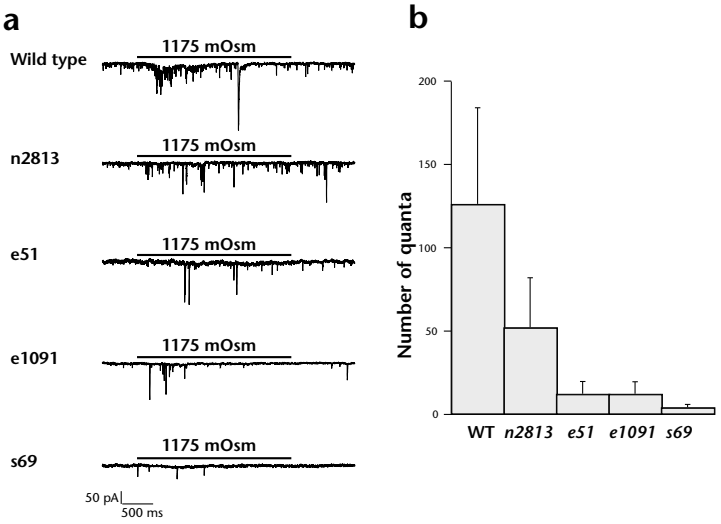

Fig. 8. Release of synaptic vesicles is reduced in response to hyperosmotic media in *unc-13* mutants. (**a**) Sample traces of postsynaptic currents before, during and after application of hyperosmotic medium to wild-type animals and *unc-13* mutants. (**b**) Total quanta released by application of hyperosmotic media in wild-type animals (n = 7) and *unc-13* mutants (n = 5).

Methods

Genetics. Nematodes were maintained as described[1] except that they were grown on the HB101 strain of E. coli. Strains used were N2 Bristol, MT7429 *unc-13*(*n2813*), MT7929 *unc-13*(*e51*), CB1091 *unc-13*(*e1091*) and BC168 *unc-13*(*s69*). The original CB51 *unc-13*(*e51*) strain contains a second mutation, *unc-122*(*n2916*), which was outcrossed to obtain MT7929.

Electrophysiology. Methods were essentially as described[19]. Briefly, animals were immobilized with cyanoacrylic glue, and a lateral incision was made to expose the ventral medial body wall muscles. Electrophysiological recordings were made in the whole-cell, voltage-clamp configuration (holding potential, –60 mV) at room temperature using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany) and digitized at 2.9 kHz via an ITC-16 interface (Instrutech, Great Neck, New York). Data were acquired by Pulse software (HEKA) run on a Power Mac 6500/225. A fire-polished electrode positioned close to the ventral nerve cord was used to stimulate evoked release using a 2-ms depolarizing current. The bath solution contained 140 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, 5 mM $MgCl_2$, 11 mM glucose and 5 mM HEPES, pH 7.2, ~330 mOsm. The pipet solution contained 120 mM KCl, 20 mM KOH, 4 mM $MgCl_2$, 5 mM *N*-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid, 0.25 mM $CaCl_2$, 4 mM NaATP, 36 mM sucrose and 5 mM EGTA, pH 7.2, ~315 mOsm. Hyperosmotic saline (1175 mOsm) was produced by adding sucrose to external medium. The current produced during the three-second application of hyperosmotic saline was integrated and summed using the Mini Analysis Program (Jaejin Software, Leonia, New Jersey, USA) to determine the net charge transfer. To compensate for differences in basal release rates, integrated currents from a three-second baseline recording were subtracted from the hyperosmotic values. Net charge transfer per quantum was determined from calcium-free minature currents for each genotype. The average quantal charge transfer was divided into the net charge transfer from hyperosmotic stimulation to determine the quantal content.

Data were subsequently analyzed and graphed with Pulsefit (HEKA), Mini Analysis (Jaejin Software) and Igor Pro (Wavemetrics, Lake Oswego, Oregon). All statistically derived values are given as mean ± s.e.

Electron Microscopy. Adult nematodes were prepared for transmission electron microscopy as described[17], with minor alterations to the protocol. Specimens were immersed in ice-cold fixative (0.7% glutaraldehyde, 0.7% osmium tetroxide in 10 mM HEPES buffer) for 1 h. Animals were then washed thoroughly in buffer, and the heads and tails were cut off in buffer. Tissue was postfixed in 2% osmium tetroxide in 10 mM HEPES buffer for 3 h. Specimens were then washed in water, stained *en bloc* in 1% uranyl acetate, dehydrated through an ethanol series, passed through propylene oxide and embedded in epoxy resin. Ribbons of ultrathin (~35 nm) sections were collected and examined on a Hitachi H-7100 electron microscope equipped with a Gatan slow-scan digital camera. Morphometric analysis was done with the public-domain software package NIH Image. For quantifying synaptic vesicles, we defined a synapse as the set of serial sections containing a discernable presynaptic density, as well as adjacent sections in which the density of synaptic vesicles was greater than the average number of vesicles per section for the whole reconstruction. Docked vesicles were defined as those vesicles located within a single vesicle radius (~30 nm) of the presynaptic plasma membrane. GABAergic synapses were recognized by their position in the ventral nerve cord and because they are polarized laterally, whereas cholinergic neuromuscular junctions are polarized dorsally. Significance values were calculated using Student's *t*-test for synaptic vesicle numbers, and the Wilcoxin rank-sum test for docked vesicle number and percentage of docked vesicles.

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