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Defects in synaptic vesicle docking in *unc-18* mutants

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Sec1-related proteins function in most, if not all, membrane trafficking pathways in eukaryotic cells. The Sec1-related protein required in neurons for synaptic vesicle exocytosis is UNC-18. Several models for UNC-18 function during vesicle exocytosis are under consideration. We have tested these models by characterizing *unc-18* mutants of the nematode *Caenorhabditis elegans*. In the absence of UNC-18, the size of the readily releasable pool is severely reduced. Our results show that the near absence of fusion-competent vesicles is not caused by a reduction in syntaxin levels, by a mislocalization of syntaxin, by a defect in fusion or by a failure to open syntaxin during priming. Rather, we found a reduction of docked vesicles at the active zone in *unc-18* mutants, suggesting that UNC-18 functions, directly or indirectly, as a facilitator of vesicle docking.

Synaptic vesicles must be tethered to sites of release and then become competent for fusion with the plasma membrane. These processes are called docking and priming, respectively[1]. The molecular processes of docking are not well understood. Priming appears to involve the formation of trans-SNARE complexes, in which the synaptic vesicle protein synaptobrevin (also called VAMP) forms a helical bundle with the plasma membrane proteins syntaxin and SNAP-25 (ref. 2). The formation of SNARE complexes is sufficient for membrane fusion *in vitro*[3], but may require other components *in vivo*[4,5].

Another protein that is involved in synaptic vesicle exocytosis is UNC-18 (ref. 6). *unc-18* mutants were first isolated in *C. elegans* on the basis of their severe locomotion defects[7]. The UNC-18 protein is similar to the yeast SEC1 protein[8], and orthologs of UNC-18 function at the synapses of fruit flies (Rop)[9] and vertebrates (Munc18-1; also known as Stxbp1, N-sec1, Rb-sec1)[10]. Genetic studies of UNC-18 in various organisms suggest that UNC-18 is required for synaptic vesicle exocytosis. For example, *unc-18* mutants in *C. elegans* are severely paralyzed and are resistant to inhibitors of cholinesterase but sensitive to acetylcholine (ACh) receptor agonists[11], implicating UNC-18 in the exocytosis of ACh. Four potential functions for UNC-18 in exocytosis have been proposed[12]: a trafficking factor for syntaxin[13], a facilitator of fusion[14], a facilitator of priming[15] or a facilitator of docking[16].

The first model proposes that UNC-18 has an indirect role in vesicle exocytosis by trafficking syntaxin to the plasma membrane. In cell culture, syntaxin is not transported to the plasma membrane unless Munc18-1 is coexpressed with the SNARE protein[13]. In yeast, the syntaxin homolog TLG2 is degraded by the proteasome unless the cognate UNC-18 homolog is present to protect it[17]. Consistent with this idea is the fact that in Munc18-1 knockout mice, syntaxin levels are reduced by half[16].

A second model suggests that UNC-18 may function in the final step of exocytosis, that is, in the fusion of the synaptic and plasma membranes. Specifically, overexpression of a mutant form of Munc18-1 in adrenal chromaffin cells alters the dynamics of the fusion pore in single-granule exocytosis[14], suggesting that the positive role for UNC-18 occurs at the fusion step after the formation of the SNARE complex. A role in fusion is consistent with the absence of synaptic vesicle fusion events at central synapses in mice lacking Munc18-1 (ref. 10). Moreover, these data are consistent with studies in yeast in which an UNC-18 homolog, Sec1p, has been shown to function after the formation of the SNARE complex[18]. However, fusion of dense core vesicles still occurs in Munc18-1 knockout mice[16].

A third model proposes that UNC-18 facilitates the priming step of synaptic vesicle exocytosis through its interaction with the SNARE protein syntaxin[15]. Syntaxin can adopt two configurations[19]. In the open configuration, the SNARE domain of syntaxin can interact with SNAP-25 and synaptobrevin. In the closed configuration, the amino terminus of syntaxin folds over the SNARE domain and occludes interactions with the other SNARE proteins[19]. Thus, syntaxin must be 'opened' for the SNARE complex to form. UNC-18 proteins from *C. elegans* and vertebrates bind syntaxin with nanomolar affinity[6]. Furthermore, syntaxin rendered constitutively open fails to bind UNC-18 in pulldown assays[19]. One possible model for UNC-18 is that binding to the closed state of syntaxin is a prerequisite for the

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opening of syntaxin; once syntaxin is in the open state, UNC-18 is released, which allows the formation of the SNARE complex[15,19]. Consistent with this model, expression of a truncated form of the yeast syntaxin homolog, Tlg2p, partially suppresses mutants of the UNC-18 homolog Vps45p[17].

A fourth model proposes that UNC-18 functions before vesicle priming and fusion to promote synaptic vesicle docking. Dense-core vesicles in Munc18-1 knockout mice fail to associate with the plasma membrane in chromaffin cells[16]. Given that dense-core vesicle exocytosis uses many of the same proteins as synaptic vesicle exocytosis[20], UNC-18 may function to promote synaptic vesicle docking as well. However, no docking defect was observed for synaptic vesicles at synapses in the hippocampus of Munc18-1 mutant mice[10], suggesting that UNC-18 function may be different for synaptic vesicle exocytosis and dense core vesicle exocytosis.

In addition to promoting release, UNC-18 has been implicated as an inhibitor of vesicle exocytosis[21]. Specifically, overexpression of UNC-18 inhibits exocytosis via its interactions with syntaxin[22]. Conversely, point mutations in UNC-18 or syntaxin that destabilize binding result in increased release of neurotransmitter[22–24]. This role for UNC-18 in exocytosis is not addressed by this study, but rather we confine our analysis to the facilitatory function of UNC-18 in exocytosis.

We tested models for UNC-18 function, and here we report that although mutations in *unc-18* result in severe defects in neurotransmission (i) UNC-18 is not required for the stabilization or trafficking of syntaxin (ii) UNC-18 is not absolutely required for fusion (iii) UNC-18 is not required to open syntaxin to facilitate priming and (iv) UNC-18 facilitates the docking of synaptic vesicles to the plasma membrane. We conclude that UNC-18 promotes, either directly or indirectly, the docking of synaptic vesicles to the plasma membrane.

RESULTS

UNC-18 is not required for neuronal development or maintenance

The *unc-18* locus was identified in screens for *unc*oordinated animals in the nematode *C. elegans*[7]. *unc-18* mutants are severely paralyzed. This paralysis could be caused by defects in the development of the nervous system. In fact, Munc18-1 knockout mice show widespread neurodegeneration after development[10]. To test whether the uncoordinated phenotype of *unc-18* mutants is caused by defects in the development or maintenance of the nervous system, we characterized the architecture of the motor neurons and the neuromuscular junctions in adult *unc-18(e234)*, *unc-18(e81)* and *unc-18(md299)* null mutant animals. *unc-18(e234)* and *unc-18(e81)* alleles are nonsense mutations[25], and *unc-18(md299)* is a multigenic deletion that removes the promoter and open reading frame of the *unc-18* gene (data not shown).

To assess neuronal outgrowth and maintenance, we analyzed the structure of the GABA nervous system using a green fluorescent protein (GFP) tag. The GABA nervous system in *unc-18(e234)*, *unc-18(e81)* and *unc-18(md299)* adult animals was indistinguishable from that of the wild type

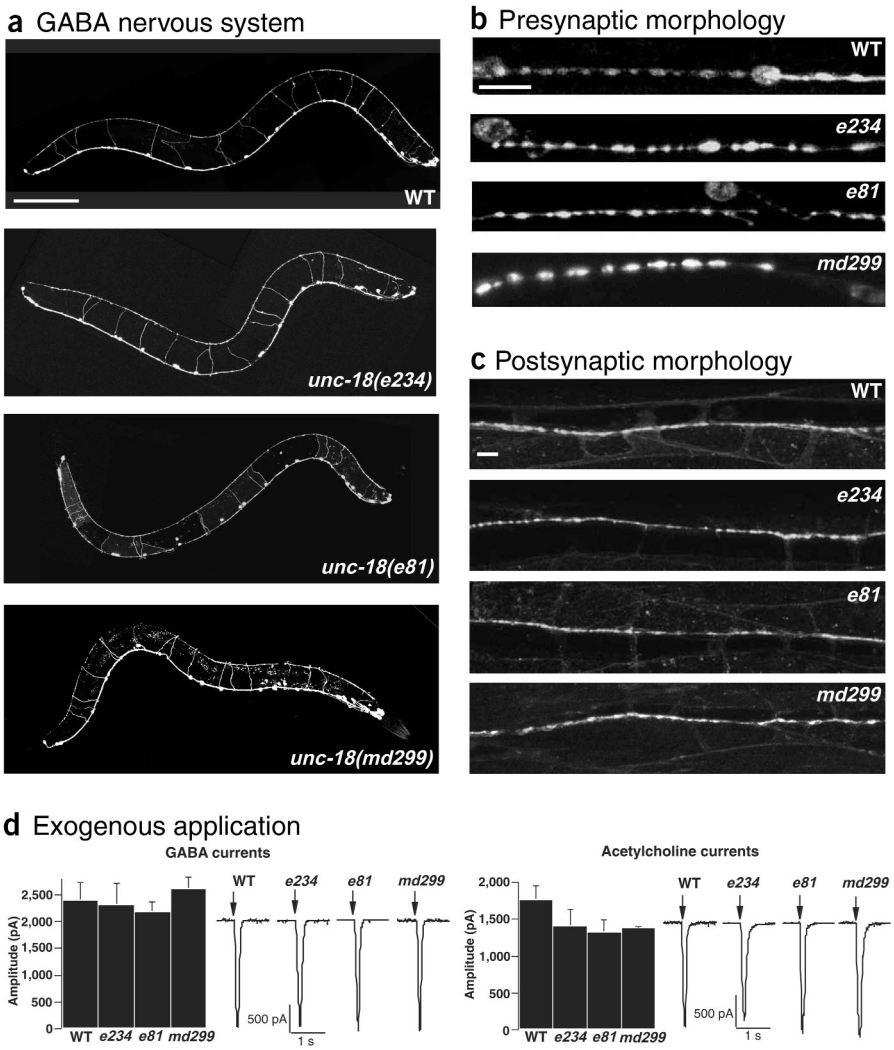

Figure 1 Normal morphology of the GABA nervous system in *unc-18* null mutants. (**a**) GABA motor neuron anatomy is normal in *unc-18* null mutants. GABA motor neurons were visualized using a GFP construct (*oxIs12*; ref. 50) in wild-type, *unc-18(e234)*, *unc-18(e81)* and *unc-18(md299)* adult worms. In all images, dorsal is up, anterior is right, and the scale bar is 100 μm. (**b**) The density and distribution of GABA synaptic varicosities is normal in *unc-18* mutants. Clusters of synaptic vesicles were visualized using a construct *(nIs52)* in which the vesicle protein synaptobrevin is fused to GFP and is expressed specifically in the GABA neurons[26]. All images are of the ventral nerve cord near the VD6 cell body; scale bar is 10 μm. (**c**) The distribution of postsynaptic GABA receptor clusters is normal in *unc-18* mutants. GABA receptor clusters in the muscles were visualized using an integrated array (*oxIs22*) expressing GFP-tagged GABA receptors in the muscle[27]. All pictures are of the dorsal nerve cord near the posterior reflex of the gonad in adult worms; the scale bar is 10 μm. (**d**) Both GABA and ACh receptors are functional in *unc-18* mutant animals. GABA or ACh (10^{-4} M) was pressure-ejected for 100 ms (arrows) onto body wall muscles of dissected wild-type and *unc-18* adults. The mean induced currents (± s.e.m.) elicited by either GABA or ACh were indistinguishable in wild-type and *unc-18* mutant worms. GABA currents are inward in our solutions at the holding potential of –60 mV.

(**Fig. 1a**). Axons extended along both the ventral and dorsal nerve cords and were stably maintained in adults.

Similarly, presynaptic nerve terminals develop and are maintained in the absence of UNC-18 (**Fig. 1b**). Briefly, synaptic vesicles were visualized by the expression of GFP-tagged synaptobrevin in GABA motor neurons[26]. The distribution of synapses as visualized by GFP-labeled clusters of synaptic vesicles in *unc-18* mutant animals was indistinguishable from that of the wild type (mean distance between synaptic vesicle clusters along the ventral nerve cord: wild type, 2.82 ± 0.20 μm, $n = 4$ animals; *unc-18(e234)*, 2.92 ± 0.14 μm, $n = 5$; *unc-18(e81)*, 2.77 ± 0.15 μm, $n = 8$; *unc-18(md299)*, 3.09 ± 0.23 μm, $n = 3$).

Furthermore, postsynaptic receptor function was normal in the absence of UNC-18. The distribution of GFP-tagged GABA receptors[27] in *unc-18* mutants was indistinguishable from wild type (**Fig. 1c**). Exogenous application of either GABA or ACh elicited robust currents from the muscles of *unc-18* null mutants, suggesting that the neurotransmitter receptors are functional in the absence of UNC-18 (**Fig. 1d**; GABA: wild type, 2403 ± 333 pA, $n = 5$; *unc-18(e234)*, 2317 ± 402 pA, $n = 5$; *unc-18(e81)*, 2181 ± 185 pA, $n = 5$; *unc-18(md299)*, 2620 ± 217 pA, $n = 3$; $P > 0.5$; ACh: wild type, 1761 ± 190 pA, $n = 8$; *unc-18(e234)*, 1410 ± 220 pA, $n = 6$; *unc-18(e81)*, 1327 ± 167 pA, $n = 6$; *unc-18(md299)*, 1385 ± 250 pA, $n = 4$; $P > 0.1$). Therefore, the primary defect associated with the loss of UNC-18 function is not due to a defect in neuronal outgrowth, synapse development or maintenance of the nervous system. These data suggest that UNC-18 is required for synaptic function.

UNC-18 is not essential to synapse function

To directly test whether UNC-18 is required for synapse function, we quantified whole-cell voltage-clamped currents from *C. elegans* neuromuscular junctions[28] (**Fig. 2a**). Stimulation of the ventral nerve cord of a wild-type animal resulted in robust postsynaptic currents (2,042 ± 82 pA, $n = 32$). However, in the *unc-18* null mutants, evoked responses were reduced to approximately 20% of the wild-type responses (*unc-18(e234)*, 491 ± 107 pA, $n = 12$; *unc-18(e81)*, 307 ± 50 pA, $n = 16$; *unc-18(md299)*, 485 ± 55 pA, $n = 28$; $P < 0.0001$). These data suggest that UNC-18 promotes the release of neurotransmitter from presynaptic nerve terminals.

The evoked currents in the *unc-18* mutants were quite small; the decreased response could have been caused by defects in the number of synaptic vesicles released or a decrease in the quantal size. Individual synaptic vesicle fusion events can be observed in *C. elegans* by recording endogenous miniature postsynaptic currents that are caused by the release of neurotransmitter from one or a few vesicles. Endogenous release events were frequent in wild-type animals (48.3 ± 3.9 fusions/s, $n = 46$; **Fig. 2b**). In

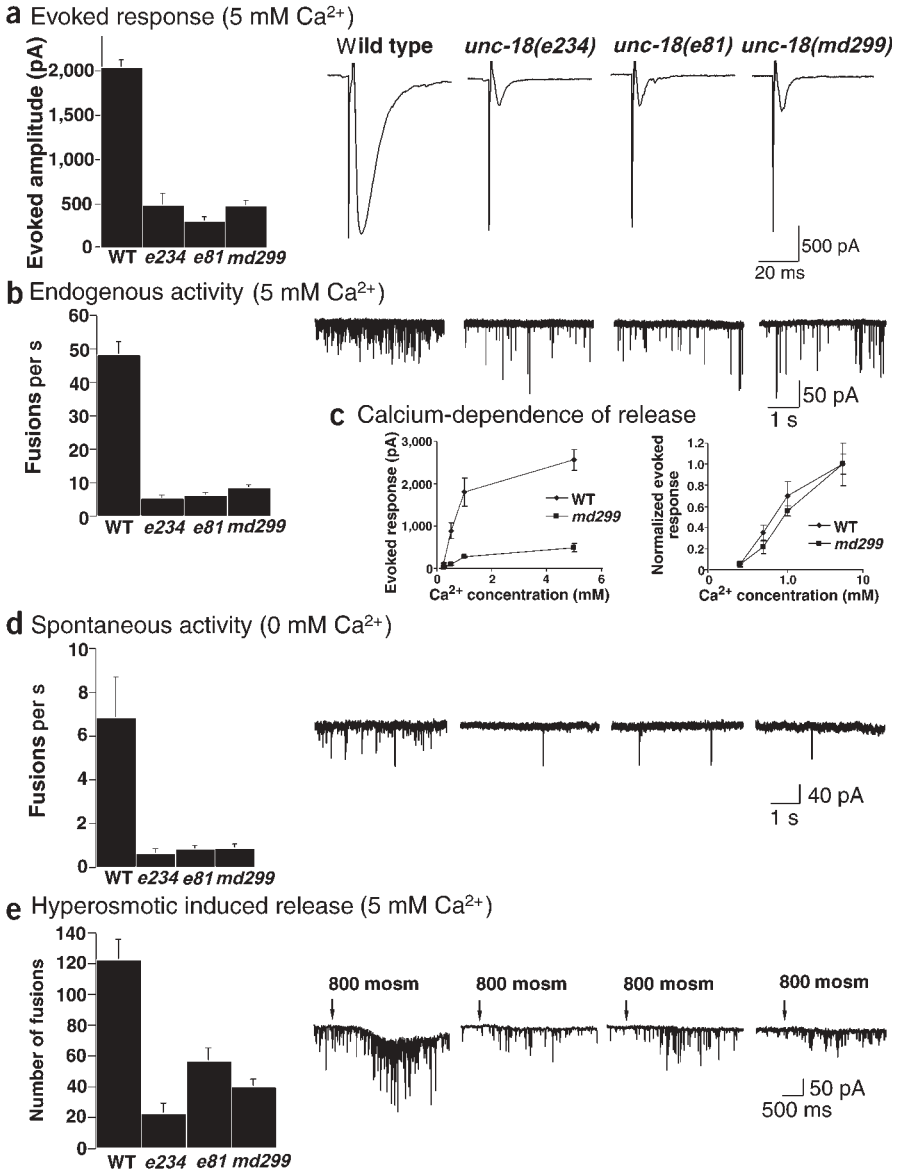

Figure 2 Neurotransmitter release is markedly reduced in *unc-18* mutant animals. (**a**) Evoked responses are reduced in *unc-18* mutants. Average evoked amplitude recorded in 5 mM Ca^{2+} plotted as mean ± s.e.m. (**b**) Endogenous release rates were reduced in *unc-18* mutants. However, synaptic vesicle fusion still occurred in the absence of UNC-18. Miniature postsynaptic currents were measured in 5 mM Ca^{2+}, and the frequency of synaptic vesicle fusions are plotted as mean ± s.e.m. Throughout this study, miniature postsynaptic currents represent release of both ACh- and GABA-containing vesicles from excitatory and inhibitory motor neurons. (**c**) The calcium-dependence of release was not altered in *unc-18* mutants. The average evoked release in *unc-18(md299)* mutants was reduced compared to the wild type (WT) at all calcium concentrations tested, but when normalized to the maximum current, the calcium-dependence of evoked release did not differ between *unc-18(md299)* and the wild type. (**d**) Spontaneous vesicle fusion events were also reduced in *unc-18* mutants. Miniature postsynaptic currents were measured in 0 mM Ca^{2+}, and the frequency of synaptic vesicle fusions is plotted as mean ± s.e.m. (**e**) The size of the readily releasable pool was reduced in *unc-18* mutants as assayed by application of hyperosmotic saline. The total number of miniature postsynaptic currents were measured during a 2-s application of hyperosmotic saline in 5 mM Ca^{2+}, and the total quanta released are plotted as mean ± s.e.m.

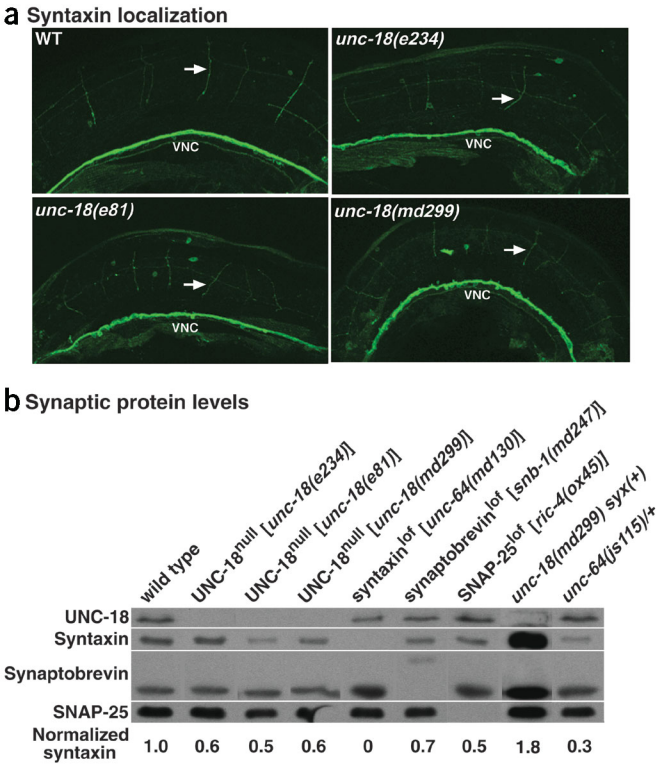

Figure 3 Syntaxin is localized but unstable in *unc-18* mutant animals. (**a**) Syntaxin localization was unaffected in *unc-18* mutant animals. Syntaxin was visualized in wild-type, *unc-18(e234)*, *unc-18(e81)* and *unc-18(md299)* animals using an anti-syntaxin antibody[48]. Syntaxin localization in *unc-18* mutant animals was indistinguishable from the wild type. Syntaxin was localized throughout the nervous system, including in the ventral nerve cord (VNC) and commissural axons (arrows). (**b**) Syntaxin levels were reduced in *unc-18* mutant animals. Equivalent concentrations of whole-worm protein extracts from wild type, *unc-18(e234)*, *unc-18(e81)*, *unc-18(md299)*, the syntaxin splicing mutant *unc-64(md130)*, the synaptobrevin hypomorph *snb-1(md247)*, SNAP-25 null mutant *ric-4(ox45)*, *unc-18(md299) syx(+)*—a strain overexpressing syntaxin from the integrated array *oxIs33* in an *unc-18* syntaxin double null mutant—and syntaxin heterozygotes *unc-64(js115)/+* were analyzed by immunoblotting with anti-UNC-18, anti-syntaxin, anti-synaptobrevin and anti-SNAP-25 antibodies. Syntaxin levels were normalized to the wild type based on the ratio of syntaxin to synaptobrevin in each genotype, except for *snb-1(md247)*, where syntaxin levels were normalized based on the ratio of syntaxin to SNAP-25.

unc-18 mutants, the frequency of vesicle fusion was decreased to about 14% of the wild-type frequency (*e234*, 5.5 ± 0.9 fusions/s, $n = 32$; *e81*, 6.3 ± 0.8 fusions/s, $n = 38$; $P < 0.0001$), but even in the complete absence of the *unc-18* locus, single fusion events still occurred (*md299*, 8.5 ± 0.8 fusions/s, $n = 28$, $P < 0.0001$). Moreover, quantal currents in *unc-18(e234)* (32.3 ± 1.9 pA, $n = 30$), in *unc-18(e81)* (37.6 ± 2.7 pA, $n = 30$) and in *unc-18(md299)* (40.5 ± 2.6 pA, $n = 40$) were not smaller but were in fact slightly larger than the wild-type quantal currents (26.6 ± 1.0 pA, $n = 34$). These results suggest that UNC-18 is required for normal levels of secretion but is not absolutely required for synaptic vesicle fusion.

The readily releasable pool is reduced in *unc-18* mutants

The decrease in release observed in *unc-18* mutants could be due to a defect in calcium sensing, in the number of vesicles primed for release, or in the probability of release of a primed vesicle. To determine whether calcium sensing was defective, we measured evoked responses over a range of external calcium concentrations. The amplitude of evoked release in *unc-18(md299)* was roughly 16% compared to that of wild type at each calcium concentration ($n > 3$; **Fig. 2c**, left). However, normalized responses were identical in the wild type and in the deletion allele *unc-18(md299)* (**Fig. 2c**, right). Thus, the calcium-dependence of release does not differ between *unc-18(md299)* and the wild type (calculated Hill coefficients were 2.8 for wild type and 2.9 for *unc-18(md299)*).

A comparative measure of vesicle priming can be determined by measuring the frequency of calcium-independent spontaneous vesicle fusions. In the wild type in 0-mM external calcium, vesicle fusion events occurred at a frequency of 6.9 ± 1.9 fusions per second ($n = 8$; **Fig. 2d**). In the *unc-18* mutants, fusion event frequency in 0-mM calcium was 11% that of the wild type (*e234*, 0.66 ± 0.2 fusions/s, $n = 4$; *e81*, 0.81 ± 0.1 fusions/s, $n = 3$; *md299*, 0.89 ± 0.2 fusions/s, $n = 4$; $P < 0.01$).

A decrease in vesicle fusion events could be caused by a decrease in the probability of fusion of a primed vesicle or by a decrease in the number of vesicles primed for release. The number of primed vesicles can be determined by application of hypertonic solution to the neuromuscular junction during recording. Hypertonic solution causes vesicles close to the plasma membrane to spontaneously fuse[29]. In the wild type, an average of 122.7 ± 13.1 ($n = 10$) vesicles were released from the plasma membrane during a 2-s application of hypertonic solution in the presence of 5mm calcium (**Fig. 2e**). In *unc-18* mutants, however, the number of quanta released by hypertonic solution was 33% of that in the wild type (*unc-18(e234)*, 23.3 ± 6.3 quanta, $n = 13$; *unc-18(e81)*, 57.5 ± 8.1, $n = 14$; *unc-18(md299)*, 40.3 ± 5.1, $n = 6$; $P < 0.0004$). To confirm that hypertonic release is not calcium-dependent, we assayed release in the absence of external calcium and observed that release in *unc-18(md299)* was 20% that of the wild type (**Supplementary Fig. 1**). Since the pool of vesicles released by hypertonic saline is thought to represent the number of primed vesicles[30], these data suggest that there is a reduction in the number of primed vesicles in *unc-18* mutants.

UNC-18 is not required for syntaxin trafficking

Synaptic vesicle priming requires the SNARE protein syntaxin[31]. Therefore, a defect in synaptic transmission in *unc-18* mutants could be due to a requirement for UNC-18 to traffic syntaxin to the synapse or to stabilize syntaxin levels. This model predicts that syntaxin should be mislocalized or absent in an *unc-18* mutant animal.

To test these predictions, we compared the localization of syntaxin in wild-type and *unc-18* mutant animals. *In situ* immunohistochemical staining showed that syntaxin is present throughout neuronal processes of wild-type animals, and that syntaxin distribution in *unc-18* mutant animals is indistinguishable from that in wild type (**Fig. 3a**). Moreover, immunogold labeling of syntaxin on ultra-thin sections showed that syntaxin is associated with axonal plasma membranes in both wild-type and *unc-18* mutant *unc-18(e234)* (**Supplementary Fig. 2**). Therefore, UNC-18 is not required for trafficking of syntaxin to the plasma membrane in neurons.

To determine whether syntaxin levels are reduced in the absence of UNC-18, we quantified syntaxin levels in wild-type and *unc-18* animals. Quantitative western analysis demonstrated that syntaxin levels are reduced roughly by half in *unc-18* mutants compared to the wild type (**Fig. 3b**). Similarly, the density of anti-syntaxin gold beads was reduced in *unc-18* mutants compared to the wild type (**Supplementary Fig. 2**). Note that mutations in other synaptic components, such as synaptobrevin and SNAP-25 also led to a 50%

reduction in syntaxin levels. These data raise the possibility that the Unc-18 phenotype could be caused by a reduction in syntaxin levels.

If the Unc-18 phenotype is caused by a 50% reduction in syntaxin, then (i) animals expressing half as much syntaxin should phenocopy *unc-18* mutants, and (ii) overexpression of syntaxin should rescue the Unc-18 phenotype. Animals heterozygous for the *C. elegans* syntaxin null mutation *unc-64(js115)* express reduced levels of syntaxin (**Fig. 3b**). However, these animals do not phenocopy *unc-18* mutants. Specifically, animals heterozygous for the syntaxin allele *unc-64(js115)* do not show an uncoordinated phenotype. Moreover, syntaxin heterozygotes had normal levels of evoked release (wild-type, 2,042 ± 82 pA, $n = 32$; *unc-64(js115)*/+, 2,030 ± 334 pA, $n = 3$; $P > 0.95$) and endogenous activity (wild-type, 48.3 ± 3.9 fusions/s, $n = 46$; *unc-64(js115)*/+, 40.1 ± 11.1 fusions/s, $n = 3$; $P > 0.6$), whereas *unc-18* mutants showed severe defects in exocytosis (**Fig. 4a**,**b**). Furthermore, overexpression of wild-type syntaxin did not rescue *unc-18* physiological defects. Specifically, overexpression of wild-type syntaxin (**Fig. 3b**, *unc-18(md299) syx(+)*) from a transgene[32] failed to rescue either evoked release (*unc-18(md299)*, 485 ± 55 pA, $n = 28$; *unc-18(md299)* syntaxin(+), 550.3 ± 76 pA, $n = 10$; $P < 0.0001$) or endogenous activity (*unc-18(md299)*, 8.58 ± 0.81 fusions/s, $n = 28$; *unc-18(md299)* syntaxin(+), 11.7 ± 2.3 fusions/s, $n = 10$; $P < 0.0001$) to wild-type levels (**Fig. 4a**,**b**, WT versus *unc-18* syx(+)). Thus, the 50% reduction in syntaxin levels in *unc-18* mutants cannot be responsible for the observed decrease in the number of primed synaptic vesicles.

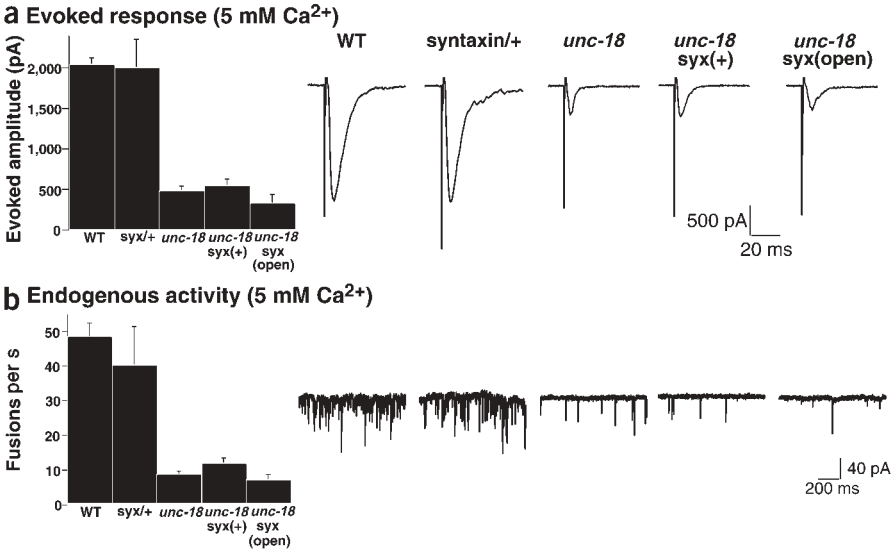

Figure 4 Overexpression of either wild-type or open syntaxin does not bypass the requirement for UNC-18 in synaptic transmission. (**a**) Evoked responses were not restored to *unc-18* mutants overexpressing wild-type syntaxin or open syntaxin. Average evoked amplitude recorded in 5 mM Ca^{2+} plotted as mean ± s.e.m. (**b**) Endogenous activity was not restored to *unc-18* mutants overexpressing wild-type or open syntaxin. Miniature postsynaptic currents were recorded in 5 mM Ca^{2+}, and the frequency of vesicle fusions plotted as the mean ± s.e.m. Syntaxin is encoded by the *unc-64* gene. In all syntaxin overexpressing strains, the endogenous locus is *unc-64(js115)*, the syntaxin null allele. 'syntaxin(+)' and 'syx(+)' signify the integrated array overexpressing wild-type syntaxin (*oxIs33*) and 'syntaxin(open)' and 'syx(open)' signify the integrated array overexpressing the open form of syntaxin (*oxIs34*).

UNC-18 is not required to open syntaxin

Formation of the SNARE complex, and thus priming, requires syntaxin to adopt an open configuration to expose its SNARE binding domain[19]. UNC-18 binding to syntaxin may be required for the conversion of syntaxin from the closed state to the open state[19], which then promotes the formation of the SNARE complex. This model predicts that expression of a constitutively open form of syntaxin should bypass the requirement of UNC-18 for neurotransmitter release.

To test this prediction, we engineered a form of syntaxin that is constitutively in the open state. Two residues in syntaxin (L165,E166) have been identified that, when mutated to alanines, render syntaxin constitutively open and unable to bind UNC-18 in pulldown assays[19]. When the corresponding mutations (L166A,E167A) are engineered in *C. elegans* syntaxin, the mutant protein also no longer binds UNC-18 in pulldown experiments[32]. Moreover, the mutant protein is able to function in place of wild-type syntaxin *in vivo*[32]. Specifically, syntaxin-null animals, expressing the open form of syntaxin, release neurotransmitter from nerve terminals at wild-type levels[32]. Neurotransmitter release via open syntaxin requires the normal presynaptic machinery, such as other SNARE proteins and calcium, but open syntaxin bypasses the requirement of proteins that function in vesicle priming, such as UNC-13 and Rim[32,33].

To test whether the open form of syntaxin could bypass the requirement for UNC-18, we expressed the open form of syntaxin in *unc-18(md299)* mutants. Overexpression of open syntaxin (3.0-fold) failed to rescue the severe paralysis associated with the *unc-18* null mutant. Consistent with the locomotion phenotype, neurotransmitter release was not restored to *unc-18* null animals expressing open syntaxin (**Fig. 4a**,**b**). Specifically, expression of open syntaxin in *unc-18* null mutants failed to rescue either evoked release (*unc-18(md299)*, 485 ± 55 pA, $n = 28$; *unc-18(md299)* syntaxin(open), 333 ± 105 pA, $n = 7$; $P < 0.0001$) or endogenous activity (*unc-18(md299)*, 8.58 ± 1.81 fusions/s, $n = 28$; *unc-18(md299)* syntaxin(open), 7.0 ± 1.5 fusions/s, $n = 6$; $P < 0.0001$) to wild-type levels. In fact, open syntaxin slightly exacerbated the phenotype of *unc-18*. Similar results were obtained using other *unc-18* mutations (*e234* and *e81*) and using another array expressing open syntaxin (*oxEx314*) (data not shown). These data suggest that the main function of UNC-18 is not to open syntaxin during synaptic vesicle priming.

UNC-18 facilitates synaptic vesicle docking

The physiological data indicate that there is a decrease in the size of the readily releasable pool of synaptic vesicles in *unc-18* mutants. Docking of synaptic vesicles with the plasma membrane is a prerequisite for vesicle priming, and thus contributes to the size of the readily releasable pool. To determine whether the distribution of synaptic vesicles is normal in *unc-18* mutants, we characterized the ultrastructure of neuromuscular junctions in *unc-18* mutants. Synaptic vesicles were abundant at presynaptic nerve terminals in *unc-18(e234)*, *unc-18(e81)* and *unc-18(md299)* synapses (**Fig. 5a**), indicating that there is no defect in the generation of vesicles. Moreover, synaptic vesicles accumulated at abnormally high levels at the synapses of *unc-18* mutants (wild-type, 394 ± 65, $n = 14$; *unc-18(e234)*, 606 ± 64, $n = 14$;

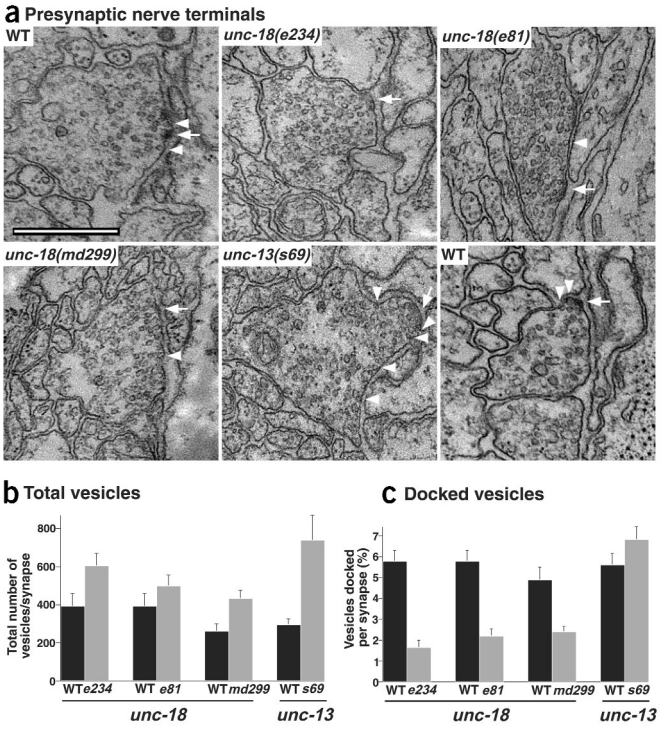

Figure 5 Fewer synaptic vesicles associate with the plasma membrane in the absence of UNC-18. (**a**) Electron micrographs of neuromuscular junctions from wild-type, *unc-18(e234)*, *unc-18(e81)*, *unc-18(md299)*, *unc-13(s69)* and *unc-13* animals. Values for the wild type vary because wild-type control animals were fixed in parallel for each mutant. Arrows indicate presynaptic specializations, and triangles indicate docked synaptic vesicles. Scale bar represents 0.5 μm. (**b**) The average number of synaptic vesicles per neuromuscular junction plotted as the mean ± s.e.m. (**c**) The proportion of docked synaptic vesicles plotted as mean ± s.e.m.

unc-18(e81), 500 ± 57, n = 18; wild-type, 263 ± 37, n = 24; *unc-18(md299)*, 434 ± 41 n = 23) (**Fig. 5b**), consistent with a defect in exocytosis.

To determine whether UNC-18 is involved in docking, we quantified the number of docked vesicles at *unc-18* synapses. The number of vesicles docked at synapses in most organisms investigated seems to be a fixed fraction of the reserve pool. Thus, as the number of vesicles in the reserve pool increases, the number of docked vesicles also increases. This increase in the number of docked vesicles can be observed in synapses of larger size[34,35] or in mutants that change the size of the reserve pool[36–40]. For this reason, the size of the docked pool is calculated as the fraction of vesicles at a synapse that are associated with the membrane. For this study, docked vesicles were defined as those in contact with the plasma membrane in sections containing a presynaptic specialization (**Fig. 5a**, triangles). For the deletion allele *md299*, wild-type and mutant sections were shuffled and scored blind. In the wild type, the proportion of vesicles in contact with the plasma membrane at a synapse is around 5–6% (**Fig. 5c**). In other mutants that accumulate synaptic vesicles to the same degree as *unc-18*, the proportion of docked vesicles remains fixed. For example, at *unc-13* mutant synapses, synaptic vesicles accumulate (wild-type, 297 ± 30, n = 19; *unc-13(s69)*, 741 ± 131, n = 13; **Fig. 5a**,**b**); however, the proportion of those vesicles contacting the plasma membrane was 6.8 ± 0.6% (n = 69 synaptic profiles)— approximately the same as the proportion in the wild type in matched studies (5.6 ± 0.5%, n = 46 synaptic profiles; **Fig. 5c**)[28,40]. In the absence of UNC-18, the number of synaptic vesicles associated with the plasma membrane was only 36% of the wild type (wild type, 5.8 ± 0.5%, n = 59; *unc-18(e234)*, 1.5 ± 0.2%, n = 65; *unc-18(e81)*, 2.1 ± 0.2%, n = 75; wild-type, 4.9 ± 0.6%, n = 24; *unc-18(md299)*, 2.4 ± 0.2%, n = 24; $P < 0.0001$). The reduction in the number of docked vesicles observed in *unc-18* mutants is not as severe as the physiological defect; clearly, not all docked vesicles are in the readily releasable pool. These data suggest that docking and priming proceed through multiple steps, one of which is affected by UNC-18.

DISCUSSION

UNC-18 is the Sec1-related protein required for synaptic vesicle exocytosis. In this study, we have explored defects in *unc-18* mutants in the nematode *C. elegans*. These studies are possible in adults because UNC-18 is not required for nervous system development in *C. elegans*. In mice, loss of UNC-18 does not cause defects in neurogenesis or in synaptic development, but the protein is required to prevent neuronal apoptosis[10]. This effect is likely to be indirect, such that loss of neurosecretion leads to programmed cell death of neurons. By contrast, in *C. elegans unc-18* mutants, the nervous system develops normally and is intact even in adult animals. Receptor clustering and function in the postsynaptic muscles also appears normal. Thus, studies of exocytosis in *unc-18* adult neuromuscular junctions have allowed us to test various models that have been proposed for UNC-18 function. Our data refute several of these models, and partially conflict with observations in other organisms.

In the first model, UNC-18 is proposed to be required for trafficking of syntaxin. In yeast, the UNC-18 homolog Vps45p is required to prevent the nearly complete degradation of the cognate t-SNARE Tlg2p[17]. In *unc-18* mutants, syntaxin levels are reduced by 50%, but this reduction cannot account for the Unc-18 phenotype. Animals heterozygous for the syntaxin null allele *unc-64(js115)* express half as much syntaxin, but they do not show defects in vesicle exocytosis. In addition, overexpression of syntaxin in an *unc-18* mutant animal does not ameliorate the UNC-18 electrophysiological phenotype. In tissue culture cells, UNC-18 is required for the trafficking of syntaxin from the Golgi apparatus to the plasma membrane[13]. However, in *unc-18* mutants, syntaxin is distributed normally along axons and at presynaptic sites. Thus, it seems unlikely that the severe defects observed in *C. elegans unc-18* mutants are due to alterations in syntaxin trafficking.

Second, some experiments indicate that UNC-18 might function at the fusion step of exocytosis. For example, no synaptic vesicle fusions were observed in CA1 hippocampal neurons from Munc18-1 mutants[10]. Moreover, overexpression of a mutant form of Munc18-1 in adrenal chromaffin cells altered the dynamics of the fusion pore in granule exocytosis[14]. In contrast to Munc18-1 mutant mice, our data show that synaptic vesicle fusion still occurs in the absence of UNC-18. Furthermore, the observed reduction in vesicle exocytosis in *unc-18* mutants can be attributed to a decreased pool of primed vesicles. The persistence of fusion events in *C. elegans unc-18* mutants parallels the observations from chromaffin cells of Munc18-1 null mice in which dense-core granule fusion events still occur[16].

A third model proposes that UNC-18 facilitates the priming step of synaptic vesicle exocytosis by promoting the open configuration of syntaxin and thus allowing the formation of the SNARE complex[15,19]. We tested this model by determining whether a constitutively open form of syntaxin could bypass the requirement for UNC-18. However, open syntaxin did not suppress the *unc-18* mutant phenotype, indicating that UNC-18 is not required to stabilize the open

configuration of syntaxin during priming.

A fourth model proposes that UNC-18 functions before vesicle priming and fusion to promote synaptic vesicle docking. Dense-core vesicles in Munc18-1 knockout mice fail to associate with the plasma membrane in chromaffin cells[16]. Given that dense-core vesicle exocytosis uses many of the same proteins as synaptic vesicle exocytosis[20], UNC-18 may function to promote synaptic vesicle docking as well. However, no docking defect was observed for synaptic vesicles at synapses in the hippocampus of Munc18-1 mutant mice[10], suggesting that UNC-18 function may be different for synaptic vesicle exocytosis and dense-core vesicle exocytosis. Our data conflict with this observation: the fraction of synaptic vesicles docked at the plasma membrane was reduced in *unc-18* mutants. To resolve this discrepancy between the mouse and *C. elegans* synaptic vesicle docking data, we will require a better understanding of the mechanisms of docking in these two organisms. We conclude that UNC-18 promotes, either directly or indirectly, the docking of synaptic vesicles to the plasma membrane.

Models for UNC-18 function

Docking has been largely defined by morphological criteria; that is, docked vesicles are identified as those that are adjacent to the plasma membrane at the active zone in electron micrographs. There is strong physiological evidence for the priming and fusion steps in exocytosis, but the docking step has depended on this relatively weak ultrastructural definition. In concrete terms, it has not been clear whether docked vesicles in electron micrographs are a pool of vesicles engaged in distinct molecular interactions or simply vesicles forced against the membrane by the high density of vesicles in the reserve pool. Reduced docking in *unc-18* mutants indicates that docked vesicles represent a pool of vesicles that can be influenced by loss of particular proteins, specifically UNC-18. However, our data do not address whether the role of UNC-18 in docking is direct or indirect.

In the simplest model, UNC-18 is directly involved in docking, by interaction with other tethering proteins. Since UNC–18 is a cytosolic protein, these tethering proteins must be localized on both the vesicular and plasma membranes. An obvious interacting protein on the plasma membrane is syntaxin, and data from yeast indicate that UNC-18 could act in SNARE preassembly[41]. However, data from *Drosophila melanogaster* and squid indicate that syntaxin is not required for docking[31,42,43]. Furthermore, additional factors at the plasma membrane would be required, since UNC-18 and syntaxin are not restricted to active zones[8,44,45] . On the synaptic vesicle, potential targets include the Rab proteins, which are implicated in vesicle docking and exhibit genetic interactions with the Sec1-related proteins[46]. In *C. elegans*, however *rab-3* mutants do not phenocopy *unc-18* mutants[47], suggesting either that Rab proteins are redundant or that UNC-18 is required for Rab3-independent processes at the synapse. Although synaptotagmin has not been shown to physically interact with UNC-18, it has been implicated in docking[39] and may eventually be shown to act in concert with UNC-18. Finally, UNC-18 physically interacts with a number of other proteins including Mint, Doc2 and granuphilin[6].

The data presented here could also support an alternative model in which UNC-18 has an indirect role in docking. One can imagine many scenarios; for example, UNC-18 could be involved at a post-fusion stage by aiding in the disassembly of SNARE complexes. Specifically, UNC-18 could bind and protect syntaxin after N-ethylmaleimide–sensitive factor (NSF)-mediated disassembly of the cis-SNARE complex. This binding could prevent the reformation of the cis-SNARE complex once these proteins are separated but while they are still in the plasma membrane. In *unc-18* mutants, functional syntaxin would be depleted because it is tied up in cis-SNARE complexes. This model is intellectually appealing because it takes into account the tight physical interaction between UNC-18 and syntaxin. In this model, the lack of free SNARE proteins either on the vesicle or on the plasma membrane would interfere with the ability of vesicles to dock with the plasma membrane.

METHODS

Genetics. Strains were maintained using standard methods as described previously[7]. The Bristol N2 strain was used as the wild type. The *unc-18* alleles *e234* and *e81* contain point mutations that result in premature stop codons at amino acid positions 141 and 528, respectively[25]. The viable *unc-18* deficiency allele *md299* contains a large (>6 kb) deletion. This deletion does not remove the *3′* end of the gene, but does delete the *unc-18* open reading frame and promoter. In addition, it removes the promoter of the syntrophin gene, which lies just upstream of *unc-18* (data not shown). The strains NM464 (genotype *unc-64(md130)*III), NM204 (genotype *snt-1(md290)*II), NM467 (genotype *snb-1(md247)*V) and NM979 (genotype *unc-64(js115)/bli-5(e518)*III) have been previously described (for details, see **Supplementary Methods** online). The *ric-4(ox45)* strain is EG45, and the *ox45* mutation disrupts the gene encoding the *C. elegans* SNAP-25 homolog (J. Lee, Y. Lee, J. Rand, M.L. Nonet & B.J. Myer, unpublished data). The syntaxin(+) strain is EG1986 *oxIs33*[*unc-64(+)*; *Punc-122*:GFP] I; *unc-64(js115)* III and the syntaxin(open) strain is EG1985 *oxIs34*[*unc-64*(L166A,E167A); *Pmyo-2*:GFP] *unc-64(js115)* III described previously[32]. The *unc-18(md299)* syntaxin(+) strain is EG2768 *oxIs33* I; *unc-64(js115)* III; *unc-18(md299)* X. The *unc-18(md299)* syntaxin(open) strain is EG2767 *oxIs34 unc-64(js115)* III; *unc-18(md299)* X.

Electrophysiology and worm dissection. Electrophysiological methods were performed as previously described[28,40] (for details, see **Supplementary Methods** online). The hyperosmotic saline, provided by addition of sucrose to a final osmolarity of 800 mosm, was applied by pressure-ejection. In this assay, individual quanta can be distinguished; thus, the size of the readily releasable pool was calculated as the total number of discrete miniature postsynaptic currents measured during a 2-s application of hyperosmotic saline in 5 mM Ca^{2+}.

Syntaxin *in situ* immunohistochemistry. Immunohistochemistry was performed on dissected wild-type and *unc-18* mutant worms fixed in 4% paraformaldehyde made in phosphate buffered saline (PBS). Fixed animals were stained with a syntaxin antibody[48] diluted 1:8,000 followed by a goat-anti rabbit Alexa Fluor 488 secondary antibody. The preparations were imaged on a Biorad confocal microscope.

Electron microscopy. Adult nematodes were prepared for transmission electron microscopy as described previously[40]. Ribbons of ultrathin sections (35 nm) were imaged using a Hitachi H-7100 electron microscope. Images were quantified using NIH Image software. A synapse was defined as a set of serial sections containing a presynaptic specialization and each flanking section containing more than one synaptic vesicle. Docked synaptic vesicles were defined as vesicles contacting the plasma membrane on the side of the axon with a presynaptic specialization. In all cases mutants and wild-type controls were fixed in parallel. For *unc-18(md299)*, micrographs were shuffled with micrographs from the wild-type control and scored blind.

Western blots. For western analysis, protein lysates were collected from mixed stage populations, separated by SDS-PAGE, transferred to nitrocellulose and probed with specific antibodies (for detailed protocols, see **Supplementary Methods** online). Blots were blocked for 1 h at room temperature, incubated in primary antibody overnight at 4 °C, and antibodies were detected using ECL. SNAP-25 was detected with affinity-purified rabbit 6470 antibodies (G. Hadwiger & M.L.N., unpublished data), synaptobrevin with affinity-purified rabbit 1092 antibodies[49], syntaxin with affinity-purified rabbit 939 antibodies[48] and UNC-18 with rabbit 601 antisera at 1:7,000[45] (a kind gift of J. Duerr & J. Rand). Intensities were quantified using NIH image.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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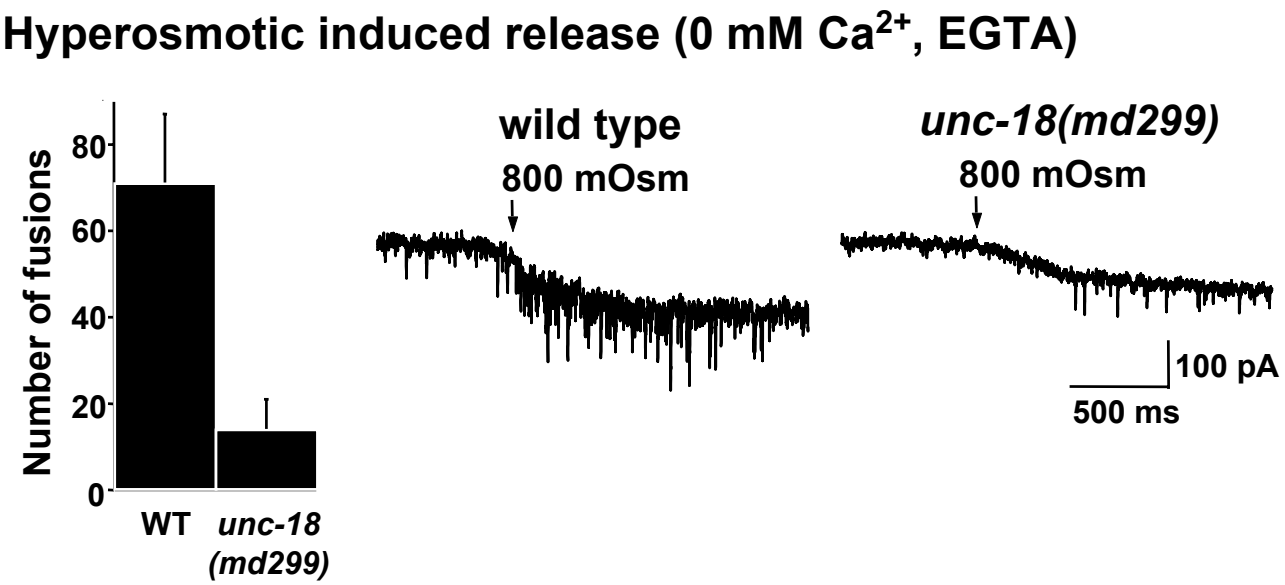
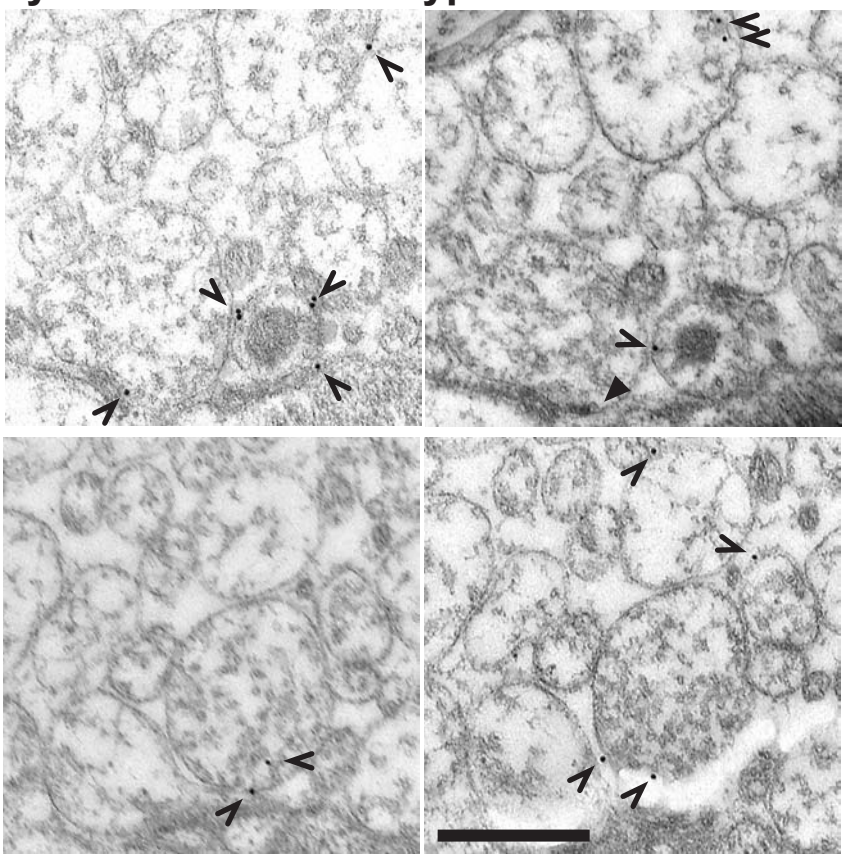
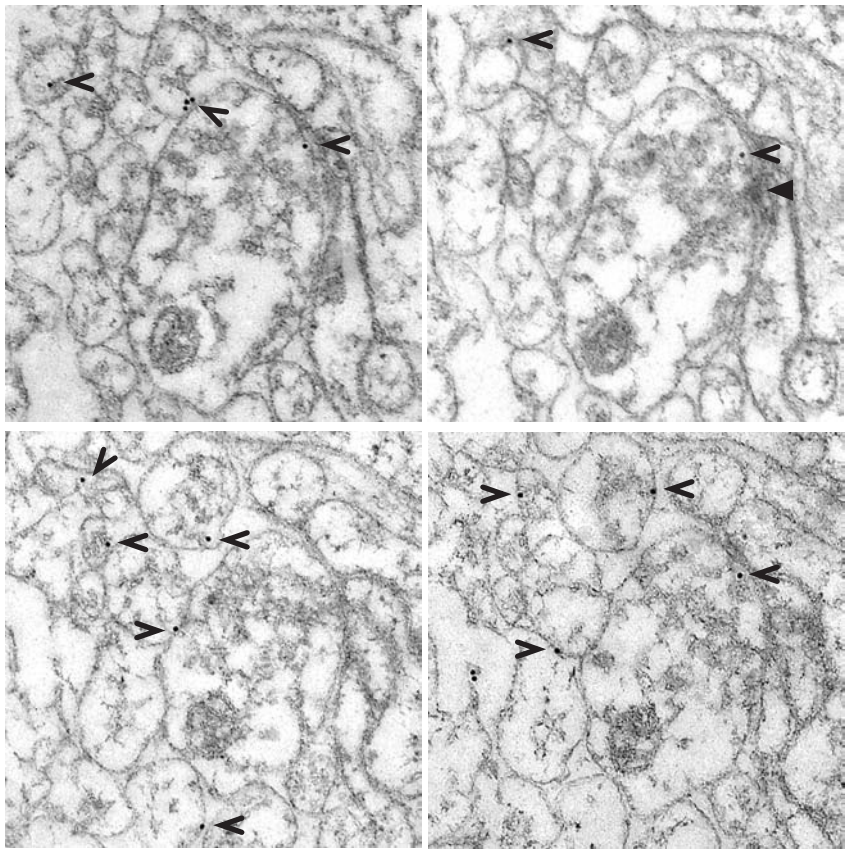

Hyperosmotic induced release (0 mM Ca2+, EGTA)
Number of fusions
80
60
40
20
0
WT
unc-18
(md299)
wild type
800 mOsm
unc-18(md299)
800 mOsm
100 pA
500 ms

Supplementary Fig. 1. The size of the readily releasable pool is reduced in *unc-18(md299)* mutants as assayed by application of hyperosmotic saline in 0 mM external calcium. The total number of miniature postsynaptic currents were measured during a 1.5 second application of hyperosmotic saline in 0 mM Ca^{2+} in the presence of 5 mM EGTA and the total quanta released plotted as mean ± SEM (n = 4 and 3 for wild-type and *unc-18(md299)*, respectively). The baseline deflection observed after application of hyperosmotic saline is due to current leak in the muscle.

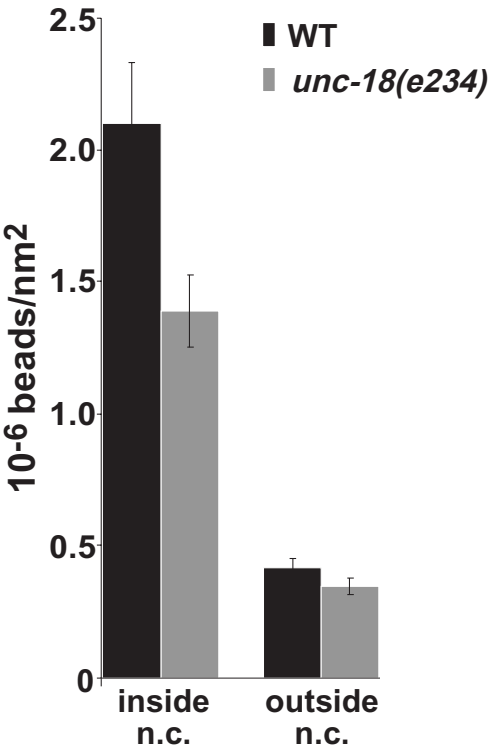
a Syntaxin in the wild type



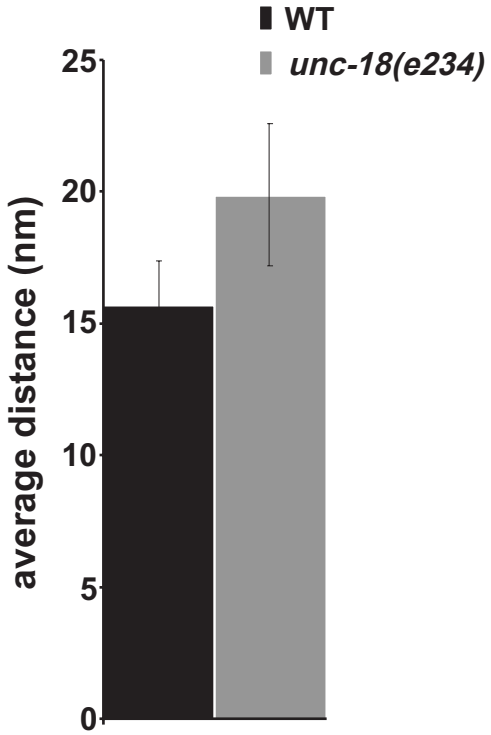
b Syntaxin in *unc-18(e234)*



c Bead density



d Distance beads are from the plasma membrane



Supplementary Fig 2. Syntaxin is not mislocalized from axonal plasma membrane in the absence of UNC-18. (**a** and **b**) Electron micrographs of neuromuscular junctions from wild-type and *unc-18(e234)* animals stained with anti-syntaxin antibody a939[1] then 15nm colloidal gold conjugated secondary antibody. In each panel, a column of micrographs represent sections in series; the closed arrowheads indicate a synapse, the open arrowheads label gold particles and the bar equals 500nm. For immunoEM analysis, Bristol N2 and EN666[*unc-18(e234)*] hermaphrodites were prepared for post-embedded immunolabeling using a protocol adapted from studies of other invertebrates[2]. Specifically, young adults were immobilized in *Escherichia coli* by high pressure freeze, fixed in anhydrous methanol with 4% paraformaldehyde and 1.5% uranyl acetate at –90^{O}C, infiltrated at –45^{O}C with lowicryl HM20 (Polyscience Inc.) which was then polymerized by UV exposure. Ultrathin sections (~40nm) were collected, incubated with the a939 antibody, labeled with gold colloidal suspensions of 15nm diameter coupled to GAR-IgG (BBInternational) following the manufactures recommendations (Aurion) and then viewed with a Philips CM20 electron microscope. (**c**) The anti-syntaxin antibody localized gold particles to axonal processes of wild-type and *unc-18(e234)* animals. For both strains, the bead density was calculated in each section by dividing the number of beads inside or outside the nerve cord by the area of the nerve cord or that of the surrounding tissue, and then plotted as the average density (10^{-6} beads/nm^2) ± SEM (wild-type, inside 2.1 ± 0.2, outside 0.4 ± 0.04 n = 48 sections; *unc-18(e234)*, inside 1.4 ± 0.1, outside 0.3 ± 0.03 n = 28 sections). (**d**) More specifically, the anti-syntaxin antibody localized gold particles to the plasma membrane of wild-type and *unc-18(e234)* neurons. The distance of gold particles from the plasma membrane within neurons was measured then plotted as the average distance ± SEM

(wild-type, 15.7 ± 1.7nm n = 277 beads; *unc-18(e234)*, 19.8 ± 2.7nm n = 195 beads). The theoretical distance between the gold particle coupled to a secondary antibody and the antigen recognized by a primary antibody is roughly 30nm. Thus, the accumulation of gold particles within 30nm of the plasma membrane in *unc-18(e234)* demonstrates that syntaxin is localized to the plasma membrane in the absence of UNC-18.

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Supplementary Methods

Strains

The strains NM464 (genotype: *unc-64(md130)*III), NM204 (genotype: *snt-1(md290)*II), NM467 (genotype: *snb-1(md247)*V) and NM979 (genotype: *unc-64(js115)/bli-5(e518)*III) have been previously described[1–3].

Electrophysiology

Electrophysiological methods were performed as previously described[4,5]. Animals were immobilized with a cyanoacrylic glue and a lateral incision was made to expose the ventral medial body wall muscles. Electrophysiological recordings from muscles were made in the whole-cell voltage-clamp configuration (holding potential –60 mV) at room temperature (21^{O}C) using an EPC-9 patch-clamp amplifier (HEKA) and digitized at 2.9 kHz via an ITC-16 interface (Instrutech). Data were acquired by Pulse software (HEKA) run on a Power Mac 6500/225. The bath solution contained NaCl 150 mM, KCl 5 mM, $CaCl_2$ 5 mM, $MgCl_2$ 1 mM, glucose 10 mM and HEPES 15 mM, pH 7.35, ~340 mOsm. The pipette solution contained: KCl 120 mM, KOH 20 mM, $MgCl_2$ 4 mM, (N-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid) 5 mM, $CaCl_2$ 0.25 mM, NaATP 4 mM, sucrose 36 mM, EGTA 5 mM, pH 7.2, ~315 mOsm. Subsequent analysis and graphing were performed using Pulsefit (HEKA), Mini Analysis (Synaptosoft) and Igor Pro (Wavemetrics).

Electron microscopy

Adult nematodes were prepared for transmission electron microscopy as described previously[6]. Specimens were fixed in 0.7% glutaraldehyde, 0.7% osmium tetroxide in 10 mM HEPES buffer for 1 hour then washed in buffer. Next, the animals' heads and tails were removed, the tissue postfixed in 2% osmium tetroxide in 10 mM HEPES buffer for 3 hours and then washed extensively in water. Specimens were then stained *en bloc* in 1% uranyl acetate, dehydrated with ethanol, passed through propylene oxide and embedded in epoxy resin.

Syntaxin *in situ* immunohistochemistry

Immunohistochemistry was performed on dissected wild-type and *unc-18* mutant worms fixed for 30 minutes in 4% paraformaldehyde made in phosphate buffered saline (PBS). Following three washes in PBS containing 0.1% Triton X (PTX), the preparation was treated with PTX containing 0.1% bovine serum albumin (PBTX) for 1 hour. A syntaxin antibody[7] was applied overnight at 4 ºC at a dilution of 1:8000 in PBTX. Following several washes in PBTX, a goat-anti rabbit Alexa Fluor 488 secondary antibody was applied for 2 hours at a dilution of 1:10,000 in PBTX. The preparations were imaged on a Biorad confocal microscope.

Western Blots

Worms were grown on NMG plates and harvested when the food supply was exhausted. These cultures are rich in newly hatched L1 larvae and yielded 40-80 µl of packed worms. Worms were collected and rinsed two times with water, and frozen at minus 80^{O}C until use. Worms were quick thawed and resuspended in 5 to 10 volumes of 360 mM sucrose, 12 mM HEPES, with a protease inhibitor cocktail (1 µg/ml pepstatin A, 1 µg/ml Leupeptin, 1 µg/ml Aprotinin, 0.1 mM PMSF), sonicated on ice four times with a 5 second burst using a 1/8 inch microtip at full power. The lysed worms were spun at 16000 g for 15 minutes to pellet the cuticle, nuclei and other large debris. The supernatant was typically in the range of 3 mg/ml protein. Approximately 25 µg of extract was diluted into SDS sample buffer, boiled for 5 minutes and separated on a 13.5% SDS-PAGE gel. Gels were transferred to nitrocellulose using a semi-dry transfer cell (biorad), 48mM tris, 39 mM glycine, 20% methanol, 1.3 mM SDS pH9.2. Transfer was assessed by staining in 0.2% ponceau S, and destaining in 1 mM NaOH. All incubations and washes were done in 1 X PBS, 0.5% Tween 20 and 5% powdered milk. Blots were blocked for 1 hour at room temperature, incubated in primary antibody overnight at 4 °C, and antibodies were detected using ECL. SNAP-25 was detected with affinity-purified rabbit 6470 antibodies (G. Hadwiger and M.L.N., unpublished), synaptobrevin with affinity-purified rabbit 1092 antibodies[2], syntaxin with affinity-purified rabbit 939 antibodies[7], and UNC-18 with rabbit 601 antisera at 1:7000[8] (a kind gift of J. Duerr and J. Rand). Intensities were quantified using NIH image.

References

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