CAPS and syntaxin dock dense core vesicles to the plasma membrane in neurons

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Docking to the plasma membrane prepares vesicles for rapid release. Here, we describe a mechanism for dense core vesicle docking in neurons. In *Caenorhabditis elegans* motor neurons, dense core vesicles dock at the plasma membrane but are excluded from active zones at synapses. We have found that the calcium-activated protein for secretion (CAPS) protein is required for dense core vesicle docking but not synaptic vesicle docking. In contrast, we see that UNC-13, a docking factor for synaptic vesicles, is not essential for dense core vesicle docking. Both the CAPS and UNC-13 docking pathways converge on syntaxin, a component of the SNARE (soluble *N*-ethyl-maleimide–sensitive fusion protein attachment receptor) complex. Overexpression of open syntaxin can bypass the requirement for CAPS in dense core vesicle docking. Thus, CAPS likely promotes the open state of syntaxin, which then docks dense core vesicles. CAPS function in dense core vesicle docking parallels UNC-13 in synaptic vesicle docking, which suggests that these related proteins act similarly to promote docking of independent vesicle populations.

Introduction

Neurons can release small molecule neurotransmitters very rapidly in part because synaptic vesicles are docked to the membrane at active zones. Docked vesicles can immediately fuse with the plasma membrane (Zenisek et al., 2000) in response to a single action potential (Borst and Sakmann, 1996; Sabatini and Regehr, 1996). Synaptic vesicle docking is defined by morphological criteria: such vesicles can be observed directly contacting the plasma membrane in electron micrographs (Couteaux and Pecot-Dechavassine, 1970; Harris and Sultan, 1995; Schikorski and Stevens, 2001; Xu-Friedman et al., 2001; Hammarlund et al., 2007). In addition to synaptic vesicles, neurons also contain secretory vesicles, called dense core vesicles, that release neuropeptides and catecholamines (Burgoyne and Morgan, 2003). Unlike synaptic vesicles, high-frequency stimulation is required for the release of dense core vesicles (Verhage et al., 1991; Bruns and Jahn, 1995; Tandon et al., 1998).

How do neurons differentially regulate synaptic vesicle and dense core vesicle release? One possible explanation for the bashfulness of dense core vesicles is that they are not docked. Dense core vesicles are usually found in the cytoplasm and these cytoplasmic vesicles must presumably translocate to the plasma membrane before release (Zupanc, 1996). However, in at least two cases, dense core vesicles have been observed docked to the plasma membrane (Karhunen et al., 2001; Ohnuma et al., 2001) and, in one case, occupied positions along the synaptic active zone (Ohnuma et al., 2001). Furthermore, secretory vesicles in neuroendocrine cells, which are similar to neuronal dense core vesicles, do dock (Plattner et al., 1997; Steyer et al., 1997). Thus, the delayed release characteristics of neuronal dense core vesicles may not be caused by a requirement for translocation to the plasma membrane.

Alternatively, the different release characteristics of dense core and synaptic vesicles might be caused by differences in spatial organization or fusion machinery. For example, dense core vesicles and synaptic vesicles might dock at different release sites, where they might experience different calcium stimuli. In this model, dense core and synaptic vesicles must have different mechanisms for docking. At *Caenorhabditis elegans* neuromuscular junctions, synaptic vesicle docking requires syntaxin, the plasma membrane SNARE protein (Hammarlund et al., 2007). Syntaxin is also involved in docking secretory vesicles in two neuroendocrine cell types (de Wit et al., 2006; Ohara-Imaizumi et al., 2007). If syntaxin docks both synaptic vesicles and dense core vesicles in neurons, it alone cannot be responsible for their different sites of release. Additional molecules, perhaps molecules that interact with syntaxin, would be required to distinguish between synaptic and dense core vesicle docking.

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Abbreviations used in this paper: CAPS, calcium-activated protein for secretion; GABA, γ-aminobutyric acid; wt, wild type.

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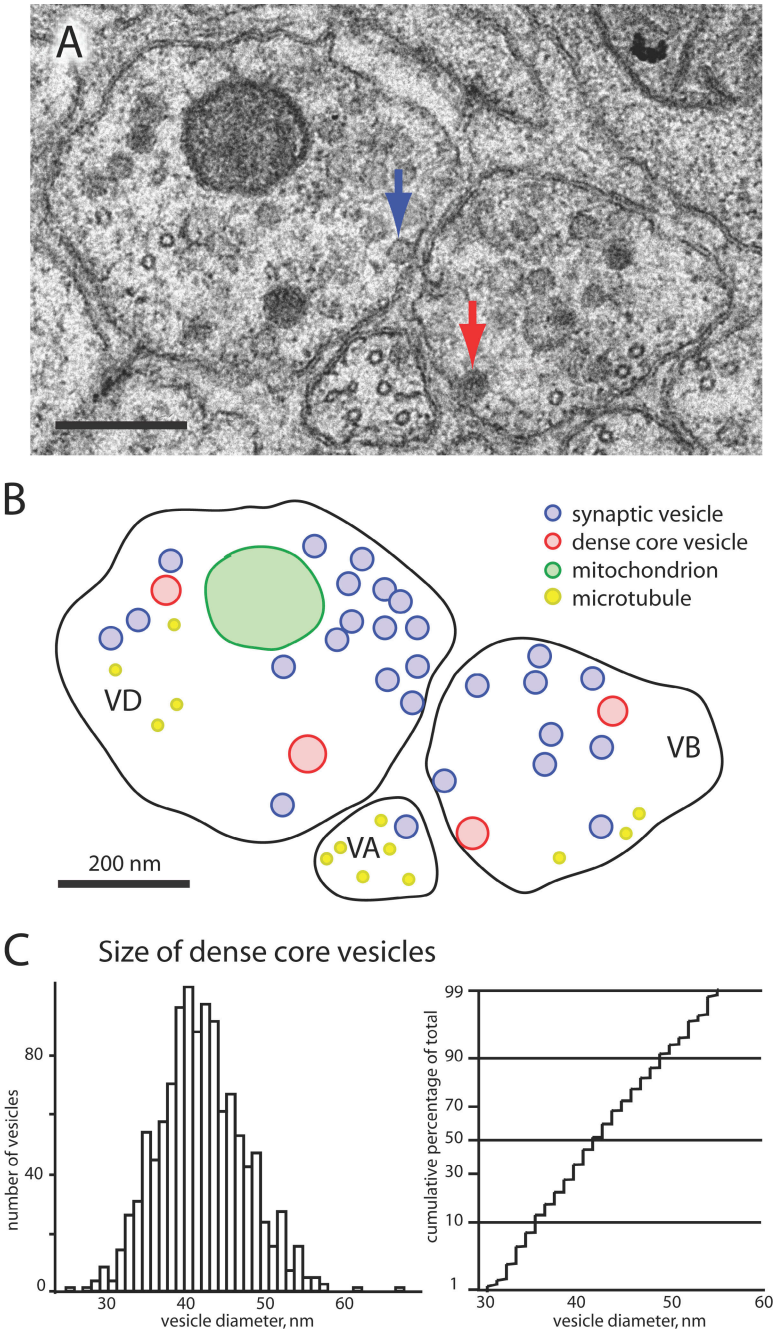

Figure 1. **Dense core vesicles dock at the neuronal plasma membrane.** (A) Electron micrograph of a portion of the ventral nerve cord from a *C. elegans* adult hermaphrodite. The blue arrow indicates a docked synaptic vesicle and the red arrow indicates a docked dense core vesicle. Bar, 200 nm. (B) A schematic depiction of the micrograph in A with some features indicated. (C) All dense core vesicle diameters displayed as a histogram (left) and a semi-log cumulative probability distribution (right). n = 990.

Two such candidates are the MUN domain proteins Unc13 and calcium-activated protein for secretion (CAPS). The Unc13 family is required for synaptic vesicle exocytosis (Aravamudan et al., 1999; Augustin et al., 1999; Richmond et al., 1999). In *C. elegans*, UNC-13 is required for synaptic vesicle docking at the active zone (Gracheva et al., 2006; Weimer et al., 2006; Hammarlund et al., 2007). Whether UNC-13 also functions in dense core vesicle docking in neurons is not known. CAPS is required for dense core vesicle exocytosis (Walent et al., 1992; Ann et al., 1997; Renden et al., 2001; Sieburth et al., 2007; Speese et al., 2007). CAPS function has not yet been assigned to any specific step of dense core vesicle exocytosis; in fact, there is some controversy over whether exocytosis should be considered to be its primary function (Speidel et al., 2005).

Here, we show that dense core vesicle docking is mechanistically distinct from synaptic vesicle docking. In *C. elegans* motor neurons, dense core vesicles dock at the plasma membrane but are excluded from the active zone. Docking of dense core vesicles in neurons, like synaptic vesicle docking, requires syntaxin. However, unlike synaptic vesicles, dense core vesicle docking requires CAPS/UNC-31 and is independent of UNC-13. A constitutively open form of syntaxin can bypass the docking defect in CAPS mutants. These data support a mechanism for dense core vesicle docking in which CAPS promotes open syntaxin and open syntaxin docks dense core vesicles. The specialized location and protein requirements for dense core vesicle docking provide a potential mechanism for the independent control of dense core vesicle release.

Results and discussion

To compare the distribution and molecular requirements for docking of dense core and synaptic vesicles, we analyzed the ventral cord motor neurons, which contain and release both vesicle types. Dense core vesicles can be distinguished from synaptic vesicles by their electron-dense appearance (Fig. 1, A and B) and are observed in both acetylcholine and γ-aminobutyric acid (GABA) neurons. These vesicles have a mean diameter of 42.5 nm and their range of diameters appears to be normally distributed around this single mean, which suggests that these vesicles are all of the same type (Fig. 1 C). Some of these vesicles are docked, appearing to directly contact the plasma membrane (Fig. 1, A and B). The fraction of dense core vesicles docked (4.5%) is roughly similar to that of synaptic vesicles (9.8%).

Are dense core vesicles clustered at synapses? The geography of vesicles at the plasma membrane can be described by their proximity to the dense projection, which marks the center of the active zone. Synaptic vesicles are packed tightly around the dense projection (Fig. 2 A), as has been observed previously (Hammarlund et al., 2007). Half of all synaptic vesicles are in profiles containing a dense projection. In contrast, only 26% of dense core vesicles are in such profiles. To encompass 50% of the dense core vesicles near a synapse, it is necessary to extend 140 nm (five profiles) on either side of the dense projection (Fig. 2 B). Furthermore, the density of synaptic vesicles in the cytoplasm is highest in profiles with the dense projection, but the density of dense core vesicles is fairly constant in all sections irrespective of the distance from the neuromuscular junction (Fig. 2, blue and red lines). Thus, dense core vesicles are only slightly enriched at synapses or are evenly distributed along the motor axon and a substantial fraction of dense core vesicles are found quite far from the dense projection.

Synaptic vesicles in *C. elegans* dock to the plasma membrane at the active zone, which flanks the dense projection at synapses (Hammarlund et al., 2007). Similar to the total synaptic vesicle population, half of all docked synaptic vesicles are found in profiles that contain a dense projection (Fig. 3 A, blue bar). This is not simply caused by increased surface area at varicosities; docked synaptic vesicles are more densely packed near the dense projection (Fig. 3 A, blue line). Docked dense core vesicles are more evenly distributed along the axon rather than densely clustered near the dense projection. In fact, profiles with a dense projection are somewhat depleted of docked dense core vesicles (Fig. 3 B, wide bar). Correcting these data for membrane area differences did not affect this trend (Fig. 3 B, red line). To further analyze this apparent depletion, we calculated the distance from the dense projection for each individual docked synaptic and dense core vesicle (Fig. 3 C). Docked dense core vesicles are excluded from the membrane within ~150 nm of the dense projection, a region that encompasses the active zone. Thus, docking of synaptic and dense core vesicles is partitioned into distinct membrane regions, which suggests that different mechanisms may control these processes.

How do dense core vesicles dock to the plasma membrane? CAPS/UNC-31 is composed of a MUN domain, a pleckstrin homology phosphoinositide-binding domain, and a C2

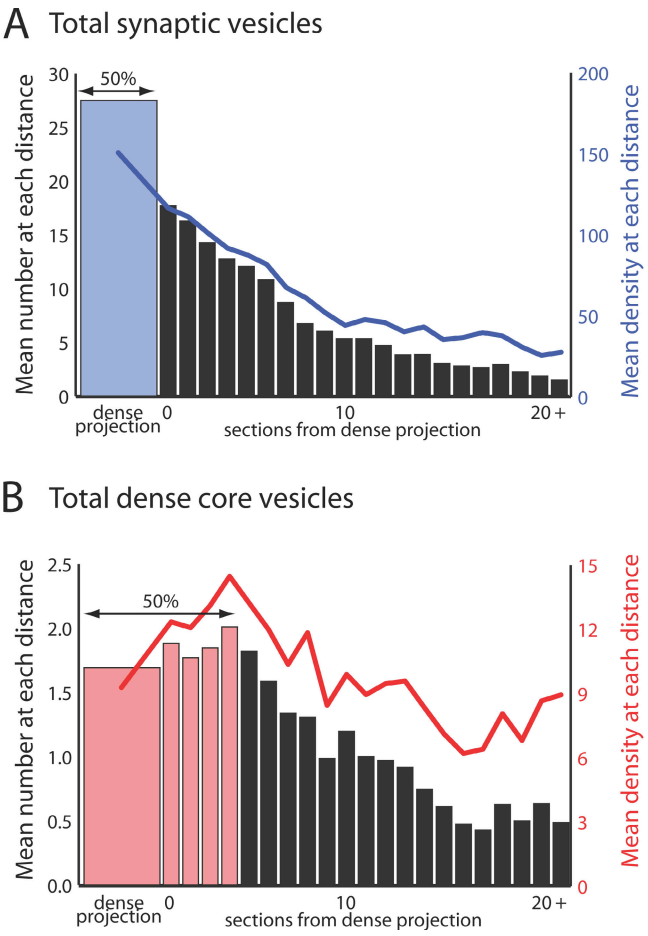

Figure 2. **Dense core vesicles do not cluster at active zones.** Each graph shows the mean number of synaptic or dense core vesicles per neuronal profile at a given number of sections from the dense projection. Colored bars show the number of profiles required to include 50% of the total number of vesicles. Lines show the vesicle density in units of vesicles per square micrometer.

phosphoinositide and calcium-binding motif (Ann et al., 1997; Speese et al., 2007). CAPS primes secretory granules in PC12 cells for calcium-stimulated release (Walent et al., 1992; Ann et al., 1997; Grishanin et al., 2004). In *C. elegans*, CAPS is essential for neuropeptide secretion but not synaptic vesicle secretion (Speese et al., 2007). It is possible that the defect in neuropeptide secretion in CAPS mutants is caused by a defect in docking of dense core vesicles. The mutation *unc-31(e928)* completely deletes the coding region for the CAPS protein (Charlie et al., 2006; Speese et al., 2007). We found that in the absence of CAPS, dense core vesicle docking is nearly eliminated (Fig. 4 A; docked dense core vesicles per profile: wild type, 0.057; *unc-31(e928)*, 0.005; $P < 0.0001$). Lack of docking is not caused by a reduction in the total number of dense core vesicles because *unc-31(e928)* mutant animals have more total dense core vesicles than the wild type (Fig. 5 B; total dense core vesicles per profile: wild type, 0.95; *unc-31(e928)*, 2.01; $P < 0.0001$). To confirm that the defect in *unc-31(e928)* in dense core vesicle docking is caused by a loss of UNC-31 rather than a potential background mutation, we performed a similar analysis on a second allele, *unc-31(u280)* (Fig. 4 A; Speese et al., 2007). No docked dense core vesicles

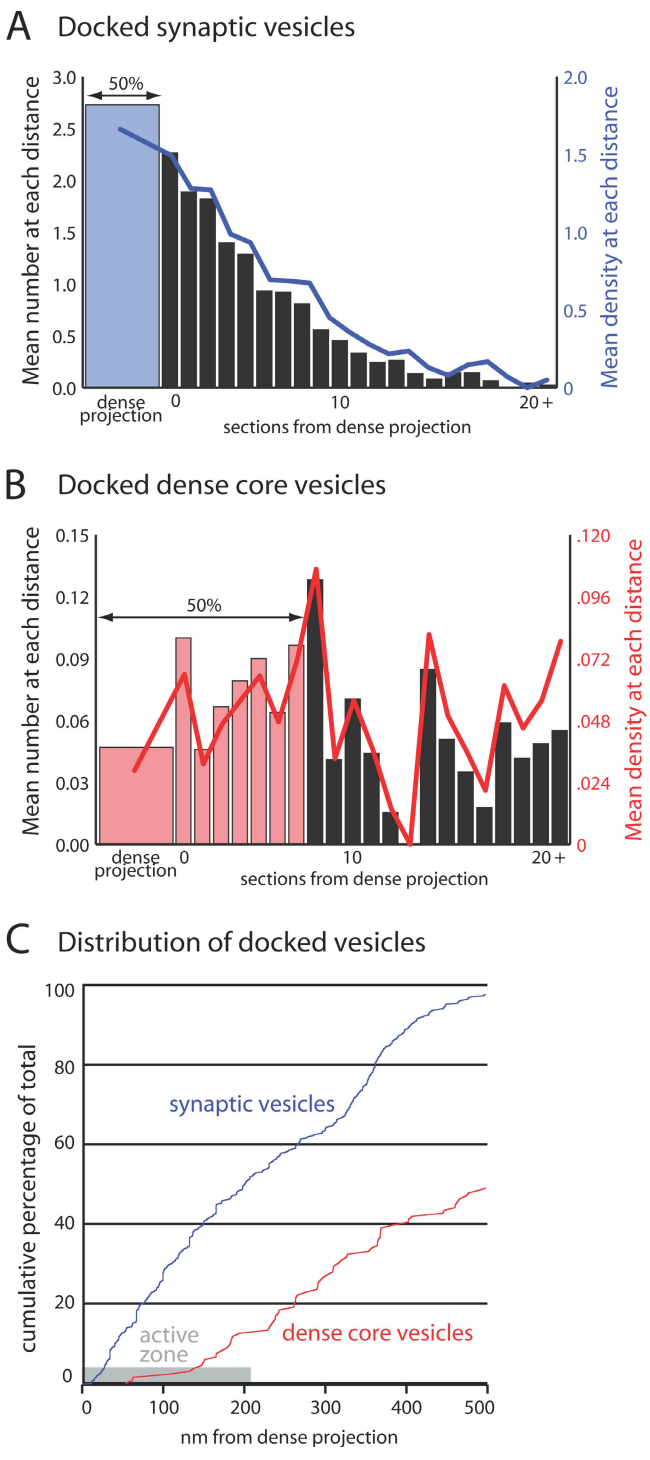

Figure 3. **Docked dense core vesicles are excluded from active zones.** (A and B) Each graph shows the mean number of docked synaptic or dense core vesicles per profile at a given number of sections from the dense projection. Colored bars show the number of profiles required to include 50% of the total number of docked vesicles. Lines show the docked vesicle density in units of vesicles per micrometer. (C) Cumulative probability of vesicle docking relative to distance from the dense projection. The active zone where synaptic vesicles dock is roughly 210 nm in radius (Hammarlund et al., 2007).

were observed in the *unc-31(u280)* allele (docked dense core vesicles per profile: wild type, 0.057; *unc-31(u280)*, 0.0; $P < 0.0001$). Furthermore, like *unc-31(e928)*, *unc-31(u280)* accumulated total dense core vesicles (Fig. 5 C; total dense core vesicles per profile: wild type, 0.95; *unc-31(u280)*, 1.19; $P = 0.0006$). The accumulation of dense core vesicles in *unc-31* mutants is consistent with the proposed function of CAPS in dense core vesicle release. Similarly, *Drosophila melanogaster* CAPS mutants have an increased number of dense core vesicles in the boutons of neuromuscular junctions (Renden et al., 2001). The near-complete lack of docked dense core vesicles in the two *unc-31* mutants demonstrates that CAPS is required for dense core vesicle docking.

Docking of synaptic vesicles in the active zone requires UNC-13 (Gracheva et al., 2006; Weimer et al., 2006; Hammarlund et al., 2007). UNC-13 is the founding member of the Unc13 class of proteins (Maruyama and Brenner, 1991). These large neuronal proteins are localized to synapses, may bind syntaxin via their MUN domains, and contain a C1 diacylglycerol-binding domain and multiple C2 phosphoinositide and calcium-binding motifs (Brose et al., 2000). Because UNC-13 shares a region of homology (the MUN domain) with CAPS (Koch et al., 2000; Basu et al., 2005) and is a vesicle docking factor, it is possible that UNC-13 is also required for dense core vesicle docking. To test whether UNC-13 functions in dense core vesicle docking, we analyzed two *unc-13* alleles: *unc-13(s69),* which affects all splice forms, and *unc-13(e1091)*, which only affects the long isoform (Kohn et al., 2000). Unlike the two CAPS alleles, most dense core vesicles are still docked in both *unc-13* alleles, which demonstrates that UNC-13 is not essential for dense core vesicle docking (Fig. 4 A). These data demonstrate that, compared with the strong effect of loss of CAPS, dense core vesicle docking is largely independent of UNC-13.

However, both *unc-13* alleles exhibited a detectable change in dense core vesicle docking. *unc-13(s69)* had 59% as many docked dense core vesicles as the wild type (docked dense core vesicles per profile: wild type, 0.057; *unc-13(s69)*, 0.034; $P = 0.0249$). The decrease in docking was not caused by a decrease in vesicle biogenesis because total dense core vesicle numbers in the *unc-13(s69)* allele were increased (Fig. 5 D; total dense core vesicles per profile: wild type, 0.95; *unc-13(s69)*, 2.07; $P < 0.0001$). In contrast, *unc-13(e1091)* had an increased number of docked dense core vesicles (docked dense core vesicles per profile: wild type, 0.057; *unc-13*, 0.081; $P = 0.0093$) and no change in the total number of dense core vesicles (Fig. 5 E; dense core vesicles per profile: wild type, 0.95; *unc-13(e1091)*, 0.89; $P = 0.53$). The decrease in dense core vesicle docking specific to the *s69* allele is consistent with an observed defect in neuropeptide release, whereas alleles that affect only the long form of UNC-13 (*e1091* and *e51*) do not reduce docking or neuropeptide release (Speese et al., 2007). We are reluctant to make strong statements about the role of UNC-13 in dense core vesicle release, given the necessarily restricted dataset from ultrastructural analyses and the general paucity of dense core vesicles; however, these data suggest that UNC-13 might play an ancillary role in dense core vesicle docking and release.

Does CAPS play a role in synaptic vesicle docking? *unc-31(e928)* mutants exhibit normal active zone synaptic vesicle docking (Fig. 4 B; active zone docked synaptic vesicles per profile: wild type, 1.92; *unc-31(e928)*, 1.91; $P = 0.96$). *unc-31(u280)* mutants had a small but significant decrease in synaptic vesicle docking at the active zone (active zone docked synaptic vesicles

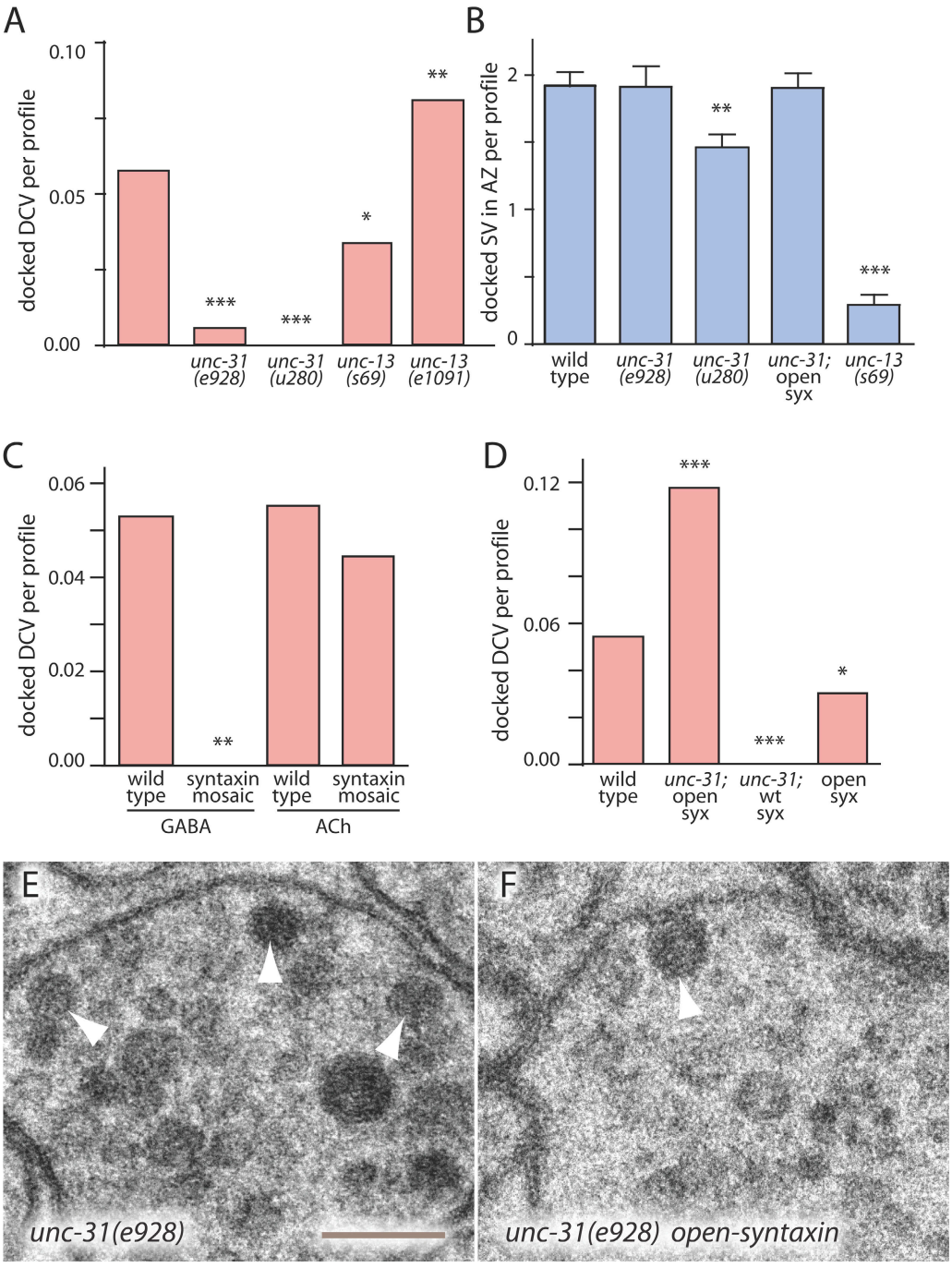

Figure 4. **CAPS/UNC-31 functions with syntaxin to dock dense core vesicles.** (A) UNC-31 is required for docking of dense core vesicles. (B) UNC-31 is not required for docking synaptic vesicles at active zones. (C) Syntaxin is required for docking dense core vesicles. In the mosaic strain, syntaxin is present in acetylcholine neurons but absent in GABA neurons. (D) Open syntaxin bypasses the requirement for UNC-31. DCV, dense core vesicle. (E) Dense core vesicles close to the plasma membrane but not docked (arrowheads) in *unc-31*. (F) A dense core vesicle that is docked (arrowhead) in *unc-31 open syntaxin*. Asterisks indicate approximate p-values (***, $P < 0.001$; **, P between 0.01 and 0.001; *, P between 0.05 and 0.01; see text for exact p-values). Error bars indicate SEM of the genotype mean. Syx, syntaxin; wt, wild type. Bars, 100 nm.

per profile: wild type, 1.92; *unc-31(u280)*, 1.47; $P = 0.006$). In contrast, eliminating UNC-13 reduced synaptic vesicle docking at the active zone to 15.6% (active zone docked synaptic vesicles per profile: wild type, 1.92; *unc-13(s69)*, 0.30; $P < 0.0001$). These new data fit well with our previous analysis of *unc-13(s69)*, which showed that only 16% of the wild-type number of synaptic vesicles docked in the active zone. In that previous study, we also analyzed *unc-13(e1091)* and found a reduction to 28% of the wild type (Hammarlund et al., 2007). Thus, CAPS/UNC-31 and UNC-13 have reciprocal roles in vesicle docking. CAPS is essential for dense core vesicle docking and has at most a minor role in active zone synaptic vesicle docking. UNC-13 is required for most synaptic vesicle docking at the active zone and has only a minor role in dense core vesicle docking.

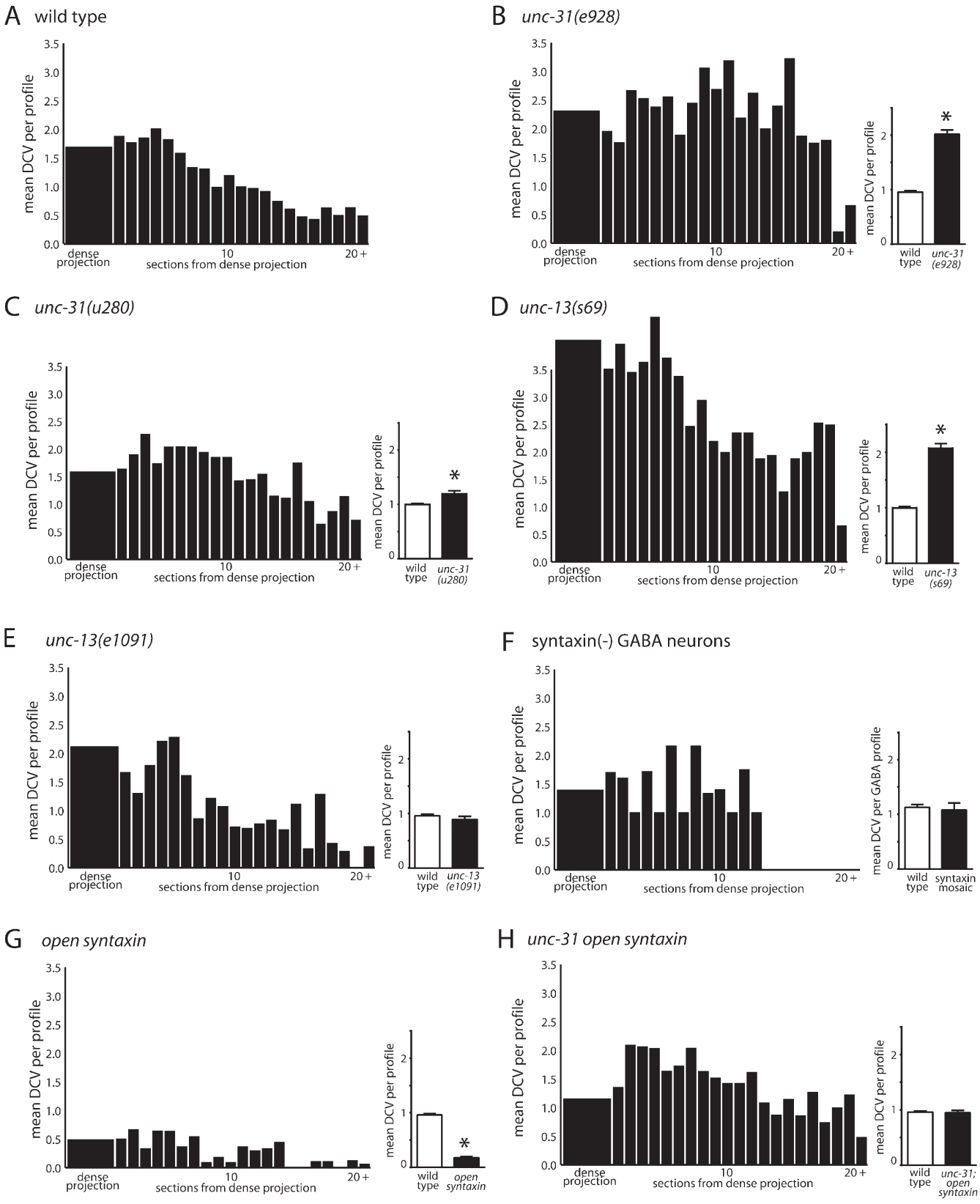

Figure 5. **Total dense core vesicle number and distribution.** (left) Dense core vesicles per neuronal profile at the given number of sections from the dense projection. (right) Mean dense core vesicles per neuronal profile for the entire reconstruction. DCV, dense core vesicle. Error bars indicate SEM. *, $P < 0.05$; see text for exact p-values.

Syntaxin is required for synaptic vesicle docking in neurons (Hammarlund et al., 2007) and secretory vesicle docking in neurosecretory cells (de Wit et al., 2006; Ohara-Imaizumi et al., 2007). We tested whether syntaxin is required for the docking of dense core vesicles by examining a mosaic strain that lacks syntaxin in the GABA motor neurons (Hammarlund et al., 2007). No dense core vesicles were docked in the syntaxin(−) neurons examined (Fig. 4 C; docked dense core vesicles per GABA neuron profile: wild type, 0.053; syntaxin mosaic, 0; P = 0.002). The lack of docking was not caused by a defect in dense core vesicle biogenesis or localization because the number and distribution of dense core vesicles in the syntaxin(−) motor neurons was similar to the wild type (Fig. 5 D; total dense core vesicles per GABA neuron profile: wild type, 1.13; syntaxin mosaic, 1.08; P = 0.59). This mosaic strain is very uncoordinated but the docking defect was not a nonspecific effect caused by paralysis. Specifically, the syntaxin(+) acetylcholine neurons had normal levels of docked dense core vesicles (Fig. 4 C; docked dense core vesicles per ACh neuron profile: wild type, 0.056; syntaxin mosaic, 0.045; P = 0.63). Together, these results demonstrate that syntaxin is required for dense core vesicle docking in neurons.

Syntaxin can adopt two conformations: a "closed" conformation, in which the amino-terminal helical region, called the "Habc" domain, binds in cis to the SNARE motif, and an "open" conformation, in which these regions do not associate (Dulubova et al., 1999). In *C. elegans*, expression of open syntaxin (Richmond et al., 2001) completely bypasses the synaptic vesicle docking defect of *unc-13* mutant animals (Hammarlund et al., 2007). These data suggest that UNC-13's function in synaptic vesicle docking is to promote the open conformation of syntaxin. If CAPS functions analogously to UNC-13, then open syntaxin should also bypass the requirement for CAPS in dense core vesicle docking. We found that dense core vesicle docking was completely rescued in *unc-31* mutants expressing constitutively open syntaxin; in fact, these animals have an increased number of docked dense core vesicles (Fig. 4 D; docked dense core vesicles per profile: wild type, 0.055; *unc-31 open syntaxin*, 0.118; P < 0.0001). Thus, open syntaxin bypasses the requirement for CAPS in dense core vesicle docking.

Importantly, the rescue of dense core vesicle docking by open syntaxin is not simply caused by overexpression of syntaxin because overexpression of wild-type syntaxin does not rescue docking in *unc-31* mutant animals (Fig. 4 D; docked dense core vesicles per profile: *unc-31*, 0.005; *unc-31 wt syntaxin*, 0.0; P < 0.0001). Open syntaxin in an otherwise wild-type background does not lead to excessive docking; in fact, such animals exhibit a slight reduction in docked vesicles (Fig. 4 D; docked dense core vesicles per profile: wild type, 0.055; *open syntaxin*, 0.030; P = 0.0139). Together, these data suggest that CAPS functions upstream of syntaxin in dense core vesicle docking by promoting the open state of syntaxin.

In addition to these conclusions, one result merits further consideration: a potential function for MUN domain proteins like CAPS downstream of syntaxin. In the strain overexpressing open syntaxin, the total number of dense core vesicles is decreased compared with the wild type (Fig. 5 E; total dense core vesicles per profile: wild type, 0.95; *open syntaxin*, 0.17; P < 0.0001). Dense core vesicles are likely depleted in this strain because open syntaxin results in increased vesicle consumption, perhaps by increasing priming, as it does for synaptic vesicles (McEwen et al., 2006). In the *unc-31 open syntaxin* double mutant, however, the total number of dense core vesicles is normal (Fig. 5 F; total dense core vesicles per profile: wild type, 0.95; *unc-31 open syntaxin*, 0.95; P = 0.91), whereas the number of docked dense core vesicles is increased compared with the wild type (Fig. 4 D). This increase in docking can be explained if dense core vesicles dock to open syntaxin but fail to fuse in the absence of CAPS. A function for CAPS in a late stage of secretory vesicle fusion has also been proposed (Hay and Martin, 1992). Note that CAPS could also function in synaptic vesicle fusion; most CAPS in *C. elegans* motor neurons colocalizes with synaptic vesicles at synapses (Charlie et al., 2006) and *unc-31* mutants exhibit a significant decrease in synaptic vesicle exocytosis (Gracheva et al., 2007).

Like CAPS, UNC-13 may play a postdocking role in vesicle fusion. Specifically, UNC-13 was proposed to act after docking in fusion of synaptic (Madison et al., 2005; McEwen et al., 2006) and secretory vesicles (Ashery et al., 2000). A postdocking function for UNC-13 in dense core vesicle fusion could account for the accumulation of docked dense core vesicles observed in *unc-13(e1091)* mutants (Fig. 4 A). Alternatively, the accumulation of docked dense core vesicles could simply be caused by the inactivity of the nervous system in this strain. Additional experiments, including direct measures of fusion, are required to tell whether CAPS and UNC-13 function after docking in dense core vesicle fusion. Our data show that CAPS and UNC-13 both function via syntaxin to dock largely different vesicle populations. However, both CAPS and UNC-13 may also function after docking, and this postdocking function may not be specific for dense core or synaptic vesicles.

Together, our experiments suggest that different protein complexes distinguish dense core vesicle docking from synaptic vesicle docking. The specificity of UNC-13 for synaptic vesicle docking and CAPS for dense core vesicle docking may provide spatial and regulatory specificity for exocytosis. These docking factors each facilitate the open conformation of the plasma membrane SNARE protein syntaxin. Open syntaxin then mediates the docking of both vesicle types.

Materials and methods

Strains

All strains were obtained from the Caenorhabditis Genetics Center unless otherwise indicated and maintained at 22°C on standard nematode growth medium seeded with HB101. The wild type was Bristol N2. Other strains used were: CB928 *unc-31(e928)* (Brenner, 1974; Speese et al., 2007), EG3405 *unc-31(u280)* (Speese et al., 2007), BC168 *unc-13(s69)* (Rose and Baillie, 1980), CB1091 *unc-13(e1091)* (Brenner, 1974; Maruyama and Brenner, 1991), EG3817 "syntaxin mosaic" *unc-64(js115); oxEx705(Punc-17:UNC-64; Pglr-1:UNC-64; Pacr-2:UNC-64)* (Hammarlund et al., 2007), EG2989 "*unc-31 open syntaxin*" *unc-64(js115) oxIs34(open syntaxin; Pmyo-2:GFP); unc-31(e928)* (this study), EG2812 "*unc-31 wt syntaxin*" *oxIs33[wt syntaxin; Punc-129:GFP]; unc-64(js115); unc-31(e928)* (this study), and EG1985 "*open syntaxin*" *unc-64(js115); oxIs34(open syntaxin; Pmyo-2:GFP)* (Richmond et al., 2001).

Electron microscopy

Previous ultrastructural analyses of acetylcholine motor neurons failed to detect dense core vesicles (White et al., 1986); however, these fixations

were not optimized for vesicle morphology. Here, we used high-pressure freezing followed by low-temperature fixation to better preserve membranes; we then reconstructed 20–30-µm segments of the VA, VB, and VD motor neurons from serial electron micrographs. Nematodes were prepared for transmission electron microscopy as described previously (Hammarlund et al., 2007). In brief, 10 young adult hermaphrodites were placed in a freeze chamber (100-µm well of type A specimen carrier) containing space-filling bacteria, covered with a type B specimen carrier flat side down, and frozen instantaneously in a high-pressure freezer (HPM 010; Bal-tec). Frozen animals were fixed in an automatic freeze substitution apparatus (Leica) with 1% osmium tetroxide and 0.1% uranyl acetate in anhydrous acetone. Temperature was kept at –90°C for 2 d, increased at 6°C/h to –20°C, then kept at –20°C for 16 h, and finally increased at 10°C/h to 20°C. The fixed animals were embedded in araldite resin after the infiltration series 30% araldite/acetone for 4 h, 70% araldite/acetone for 5 h, 90% araldite/acetone overnight, and pure araldite for 8 h. Mutant and control blocks were blinded. Ribbons of consecutive ultra thin (33-nm) serial sections were collected using an Ultracut 6 (Leica). Images were obtained with an electron microscope (H-7100; Hitachi) using a digital camera (Orius SC1000; Gatan). Serial images of the ventral nerve cord were collected at the level of the reflex of the gonad at 12,000×.

Morphometry

Image analysis was performed using ImageJ software. The VA and VB acetylcholine and the VD GABA motor neurons in the ventral nerve cord were analyzed for each animal. With the exception of the syntaxin mosaic genotype, data from each neuron type appeared similar and all data were pooled for final analysis. A neuronal profile is the cross-sectional image of an axon from a single section. Because each section is ~33-nm thick, a single profile represents data from a 33-nm slice of a particular neuron. The number of worms varied depending on the quality of the fixation and continuity of ribbons. Our analysis included strain genotype: cell, number profiles (number worms), respectively. N2: VA, 1027; VB, 1028; VD, 1119 (6). EG3817 syntaxin mosaic: VA, 167; VB, 167; VD, 166 (1). CB1091 *unc-13*: VA, 200; VB, 200; VD, 219 (1). CB928 *unc-31*: VA, 184; VB, 184; VD, 184 (1). EG2989 *unc-31 open syntaxin*: VA, 420; VB, 420; VD; 420 (3). EG2812 *unc-31 wt syntaxin*: VA, 335; VB, 340; VD, 341 (2). EG1985 *open syntaxin*: VA, 224; VB, 224; VD, 249 (1). Every vesicle was scored as either a synaptic vesicle (~30 nm and grayish), a dense core vesicle (~40 nm and black), or a large vesicle (>40 nm and clear) based on these criteria. Because these vesicle types appear somewhat similar, it is possible that a few vesicles may have been miscategorized. But because all scoring was done in parallel and blind to genotype, miscategorizing is not expected to introduce bias into the data. The number of synaptic vesicles and dense-core vesicles in each profile was counted and the diameter of each vesicle was measured. Docked vesicles were defined as having a contact zone with the plasma membrane.

Analysis

For the analysis of vesicle distribution in wild-type animals, data were collected from worms prepared in two separate fixations for synaptic vesicles and three for dense core vesicles. The distance of dense core vesicles to the dense projection was calculated by counting the number of sections between each profile and the closest dense projection where each section corresponds to ~33 nm. A difficulty arose at the ends of each reconstructed neuron: because a dense projection could lie just outside the reconstruction, the number of sections to the nearest dense projection cannot be determined. These profiles were therefore eliminated from our analysis, leaving 717 profiles and 15 synapses for VA and VB acetylcholine neuron synaptic vesicle analysis, 1,437 profiles and 27 synapses for VA and VB acetylcholine neuron dense core vesicle analysis, 458 profiles and 12 synapses for VD GABA neuron synaptic vesicle analysis, and 813 profiles and 19 synapses for VD GABA neuron dense core vesicle analysis. A total of 9,539 synaptic vesicles and 2,494 dense core vesicles were included in this analysis.

For each distance from the dense projection, the mean numbers of total and docked synaptic and dense core vesicles per profile were determined. Thus, in Figs. 2 and 3, the bar at 10 shows the mean number of vesicles per profile for all profiles that are 10 sections away from the nearest dense projection. The bar labeled dense projection shows the mean number of vesicles per profile for all profiles that contain the dense projection. Because the mean dense projection encompasses 4.85 sections, this bar is proportionally wider than the rest. The bar labeled "+" shows the mean number of vesicles per profile for all profiles more than 20 sections away from the dense projection. Total vesicle density in Fig. 2 was obtained for each distance by dividing the mean number of vesicles at that distance by the mean profile area at that distance. Docked vesicle density in Fig. 3 was calculated for each distance by dividing the mean number of docked vesicles at that distance by the mean profile plasma membrane length at that distance.

The distribution of docked vesicles (Fig. 3 C) was determined by calculating the distance between the docked vesicle and the dense projection. Thus, these data are calculated distances for each vesicle in nanometers rather than averages of vesicle counts within profiles. For vesicles in profiles with a dense projection, the distance from the dense projection to the vesicle was directly measured. For vesicles in profiles without a dense projection, the measurement (the radial distance) was made to an imaginary extension of the dense projection along the bore of the axon from adjacent sections. The longitudinal distance was estimated as the number of intervening sections multiplied by the section thickness (33 nm) and combined with the radial distance using the Pythagorean formula (Hammarlund et al., 2007). The cumulative probability was based on all measured vesicles. A straight line on a semi-log plot of the cumulative probability was consistent with normally distributed data.

For dense core vesicles, the mean number of vesicles or docked vesicles per neuronal profile was calculated by dividing the number of such vesicles summed across all profiles by the number of neuronal profiles analyzed. Error bars are not given because data are not normally distributed; p-values were calculated using χ^2. Because most profiles contain zero docked dense core vesicles, the number of docked dense core vesicles in each profile does not follow a normal distribution. We therefore compared dense core vesicle docking between genotypes using a χ^2 test with two categories: profiles with zero docked dense core vesicle and profiles with one or more docked dense core vesicles (out of 14,055 profiles, 30 contained two docked dense core vesicles and one contained three). Two-tailed p-values were computed using GraphPad Prism.

For synaptic vesicles, vesicles docked in the active zone were defined as those docked within 230 nm of the dense projection (measured linearly from the edge of the vesicle to the nearest edge of the dense projection in profiles containing a dense projection). A mean number of synaptic vesicles docked per active zone profile was determined separately for each synapse by grouping together contiguous profiles that contained a dense projection and dividing by the number of profiles containing a piece of the dense projection. The means determined for each synapse were averaged to generate a genotype mean (thus synapses with larger dense projections were not more heavily weighted). Error bars indicate SEM of this genotype mean. These means were compared using a *t* test. Two-tailed p-values and standard errors were computed using GraphPad Prism (GraphPad Software, Inc.).

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