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Absence of synaptotagmin disrupts excitation–secretion coupling during synaptic transmission

(*Drosophila*/synaptic vesicle/neuromuscular junction/Ca^{2+}/neurotransmission)

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ABSTRACT **Synaptotagmin is an integral synaptic vesicle protein proposed to be involved in Ca^{2+}-dependent exocytosis during synaptic transmission. Null mutations in synaptotagmin have been made in *Drosophila*, and the protein's *in vivo* function has been assayed at the neuromuscular synapse. In the absence of synaptotagmin, synaptic transmission is dramatically impaired but is not abolished. In null mutants, evoked vesicle release is decreased by a factor of 10. Moreover, the fidelity of excitation–secretion coupling is impaired so that a given stimulus generates a more variable amount of secretion. However, this residual evoked release shows Ca^{2+}-dependence similar to normal release, suggesting either that synaptotagmin is not the Ca^{2+} sensor or that a second, independent Ca^{2+} sensor exists. While evoked transmission is suppressed, the rate of spontaneous vesicle fusion is increased by a factor of 5. We conclude that synaptotagmin is not an absolutely essential component of the Ca^{2+}-dependent secretion pathway in synaptic transmission but is necessary for normal levels of transmission. Our data support a model in which synaptotagmin functions as a negative regulator of spontaneous vesicle fusion and acts to increase the efficiency of excitation-secretion coupling during synaptic transmission.**

Neurons transmit chemical signals through the Ca^{2+}-dependent exocytosis of synaptic vesicles. Vesicles ready for release are docked at specialized fusion sites or active zones at the presynaptic membrane, and fusion is triggered by the voltage-dependent influx of Ca^{2+}, presumably via a Ca^{2+}-sensing molecule (1). Hence, targeted vesicle docking and the Ca^{2+}-dependent fusion of these vesicles are two key features of synaptic transmission and may be important sites of synaptic regulation. Recently, many proteins have been identified in presynaptic terminals that may be involved in these processes (2) but *in vivo* analyses of their functions are largely lacking.

A candidate for a role in both vesicle docking and Ca^{2+} sensing is synaptotagmin, an integral synaptic vesicle protein (3, 4). This protein has a short domain within the vesicle lumen, a single transmembrane domain, and a large cytoplasmic domain that contains two regions with homology to the C2 domain of protein kinase C (4). As the C2 domain is known to bind both phospholipids and Ca^{2+}, synaptotagmin has been proposed to be a Ca^{2+} sensor directly mediating vesicle fusion (4, 5). Indeed, synaptotagmin has been shown to bind Ca^{2+} *in vitro* with the stoichiometry and affinity predicted for the physiological Ca^{2+} sensor (5, 6). Synaptotagmin has also been proposed to promote vesicle docking because *in vitro* it binds two proteins of the presynaptic membrane release site, syntaxin (7, 8) and neurexin (9, 10). Hence, the association of synaptotagmin and components of the release site might mediate vesicle targeting or the stabilization of docked vesicles ready for evoked exocytosis.

Several experiments have probed the *in vivo* function of synaptotagmin in synaptic transmission. First, injection of anti-synaptotagmin antibodies into PC12 cells (11) or peptide fragments of synaptotagmin into squid giant synapses (12) suppresses vesicle release. Second, genetic mutations in both *Caenorhabditis elegans* (*snt*; ref. 13) and *Drosophila* (*syt*; refs. 14 and 15) produce weak, behaviorally abnormal animals. In *C. elegans*, putative null mutants permit viability, suggesting that synaptotagmin is not required for synaptic function (13). In *Drosophila*, null mutants likewise permit limited movement but lead to lethality in early larval life (14). In viable hypomorphic mutants, decreased levels of synaptotagmin are found to dramatically suppress synaptic transmission (15, 16). Overall, these studies suggest that synaptotagmin is not absolutely required for synaptic transmission but plays a critical role in supporting adequate levels of release.

In this study, we recorded synaptic currents from embryos around the time of hatching to examine the true null phenotype. With a detailed examination of synaptic physiology at the neuromuscular junction, these studies permitted us to analyze the properties of transmission in the complete absence of synaptotagmin. In null mutants, vesicle release persists, proving that synaptotagmin is not absolutely required for evoked vesicle fusion. Moreover, the Ca^{2+} dependence of vesicle release is not changed in these mutants, suggesting either that synaptotagmin is not a Ca^{2+} sensor or that it coexists with a second, independent Ca^{2+} sensor. On the other hand, evoked vesicle release is dramatically suppressed in the absence of synaptotagmin, showing that the protein is important for synaptic transmission. As in hypomorphs (15, 16), spontaneous vesicle release is greatly increased in the absence of the protein, suggesting that synaptotagmin may be a negative regulator of release. Moreover, excitation–secretion coupling is disrupted in the absence of synaptotagmin, so that the fidelity of synaptic transmission is lost. These data support a model in which synaptotagmin is a negative regulator of vesicle fusion and acts to increase the efficiency of excitation–secretion coupling during synaptic transmission.

MATERIALS AND METHODS

Synaptotagmin Mutants. Lethal synaptotagmin mutants induced by ethyl methanesulfonate have been identified (14, 15). One of these, syt^{AD4} (14), contains a change from C to T that introduces a stop codon for the codon encoding amino acid 32 (16). The mutant protein lacks all of the regions that are evolutionarily conserved and thought to be functionally

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Abbreviations: wt, wild type; NMJ, neuromuscular junction; CNS, central nervous system; EJC, excitatory junction current; time AEL, time after egg laying.

important, including the transmembrane domain and both C2 repeats. Moreover, immunocytochemistry of homozygous first-instar larvae with a polyclonal anti-synaptotagmin antibody (see below) revealed no detectable staining either in the central nervous system (CNS) or neuromuscular junctions (NMJs). In the synaptic phenotype described here, homozygous syt^{AD4} resembles (data not shown) several homozygous deficiencies known to completely remove the synaptotagmin gene (14, 15). Thus, syt^{AD4} is unequivocally a null mutant and has been used throughout this study.

Electrophysiology. syt^{AD4}/CyO flies were outcrossed to wild type (wt; Oregon R), their Cy^+ progeny were crossed, and gastrulae were collected [3 hr after egg laying (AEL)]. Embryos were incubated at 25°C, and putative homozygous syt^- embryos were selected on the basis of delayed hatching (22 hr AEL) and behavioral abnormalities. For controls, we have used the parent chromosome used to generate syt^{AD4}, cn bw, outcrossed to Oregon R. The genotype of the animal was confirmed with anti-synaptotagmin immunocytochemistry following physiological experiments. Mature embryos or early L1 larvae (22–24 hr AEL) were dissected as reported earlier (17). All work was performed on a single identified NMJ on ventral longitudinal muscle 6 (17, 18) in abdominal segment A2. The preparation was viewed in a compound microscope fitted with differential interference contrast (Nomarski) optics and a ×40 water immersion lens. Whole-cell current recordings from muscle 6 were made with patch pipettes pulled from borosilicate glass (fiber-filled) with tips fire-polished to final resistances of 3–5 MΩ as reported earlier (17). The muscle was voltage-clamped at −60 mV. Signals were amplified by using an Axopatch-1D (Axon Instruments, Foster City, CA) patch–clamp amplifier and filtered with an 8-pole Bessel filter at 2 kHz. Data were analyzed with PCLAMP version 5.51 software (Axon Instruments). All recordings were performed in normal fly saline. The bath consisted of 135 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Tes, and 36 mM sucrose, where Tes is 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid. The Ca^{2+} concentration was modified as reported in the figures by using EGTA-buffered solutions. Ca^{2+}-free salines were made either by omitting $CaCl_2$ from the saline or by also adding EGTA with similar results. The intracellular solution consisted of 120 mM KCl, 20 mM KOH, 4 mM $MgCl_2$, 5 mM TES, 5 mM EGTA, 0.25 mM $CaCl_2$, 4 mM ATP, 0.4 mM GTP, and 36 mM sucrose. The pH of all solutions was buffered at 7.15. The muscle sheath was removed by incubation in collagenase [collagenase IV (Sigma) at 1 mg/ml for 1 min at room temperature] prior to patch-clamping.

The glutamate-gated excitatory junction current (EJC) was studied by L-glutamate iontophoresis at the developing NMJ on muscle 6 as reported earlier (17, 18). Synaptic transmission was studied by stimulating the motor nerve with a suction electrode where it exits the CNS and recording the synaptic current in the voltage-clamped muscle, as reported earlier (17). Briefly, a small segment of the motor nerve was drawn into a suction pipette and stimulated with short pulses (1 ms) of positive current. The resulting EJC was recorded in muscle 6 voltage-clamped at −60 mV. For analysis of quantal content, the size of the unitary quantal event was taken to be that of the spontaneous miniature EJCs recorded in wt or mutant embryos. This assumption was born out by amplitude histograms of evoked currents in low-Ca^{2+} saline that revealed the predicted peaks for unitary events and their multiples.

Immunocytochemistry. Dissected larvae were immunologically stained as reported (17, 18). Briefly, staged larvae were cut along the dorsal midline, pinned flat, and fixed for 30 min with 4% paraformaldehyde. Fixed larvae were probed with either a polyclonal anti-synaptotagmin antibody (diluted 1:2000; ref. 19) or a monoclonal anti-fasciclin II antibody (diluted 1:200; ref. 20), which recognizes all peripheral motor axons.

RESULTS

In *Drosophila*, null mutations in synaptotagmin result in lethality late in embryogenesis or early in postembryonic life (14, 15). We have used a null mutation (syt^{AD4}), a point mutation that removes all of the protein's functional domains (Fig. 1) and is phenotypically indistinguishable from large deficiencies that remove the *syt* locus (data not shown; ref. 15), to assay synaptic transmission in the complete absence of synaptotagmin. Such mutants can move, albeit in a sluggish and poorly coordinated manner, indicating that neuromuscular transmission persists in the absence of synaptotagmin. However, transmission at *syt*-deficient (syt^-) synapses is dramatically impaired (Fig. 2).

In wt larvae at hatching, activity within the CNS generates periodic activity in the motor neuron that results in episodes of neuromuscular transmission occurring as periodic bursts of robust excitatory currents with a peak amplitude of several nanoamperes (Fig. 2*A*). Each burst of synaptic currents underlies a single muscle contraction, and the periodicity of these bursts generates the regular peristaltic muscle contrac-

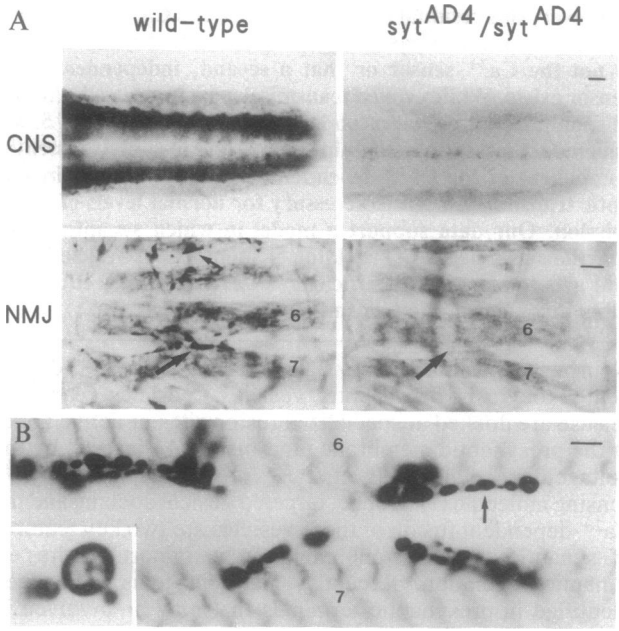

FIG. 1. Synaptotagmin encodes an integral synaptic vesicle protein expressed in *Drosophila* synapses (19) in both the CNS and NMJ. In syt^{AD4}, a stop codon introduced for the codon encoding amino acid 32 deletes all of the evolutionarily conserved protein domains (14, 16) and results in a completely null phenotype; the behavioral and physiological characteristics of syt^{AD4}/syt^{AD4} are indistinguishable from several deficiencies covering the *syt* locus. We have used syt^{AD4} homozygous mutants (syt^-) to examine synaptic transmission in the complete absence of synaptotagmin at an identified NMJ (17, 18) in mature *Drosophila* embryos at hatching (21–24 hr AEL). (*A*) In mature wt embryos, synaptotagmin is expressed at neuronal synapses in the CNS, prominently in the neuropile along the longitudinal connectives, and in the presynaptic terminals of NMJs among the somatic muscles (three arrows). The NMJ on ventral longitudinal muscle 6 (larger arrows at the bottom) has been the focus of our studies. In syt^{AD4} homozygotes, synaptotagmin expression is absent both in the CNS and at the NMJ (arrow). The morphology of the CNS and neuromusculature appears normal in syt^- embryos. (*B*) In the mature larval synapse, synaptotagmin is expressed in the boutons (arrow), the site of transmitter release. (B *Inset*) A single bouton at higher resolution. (Bars = 5 μm.)

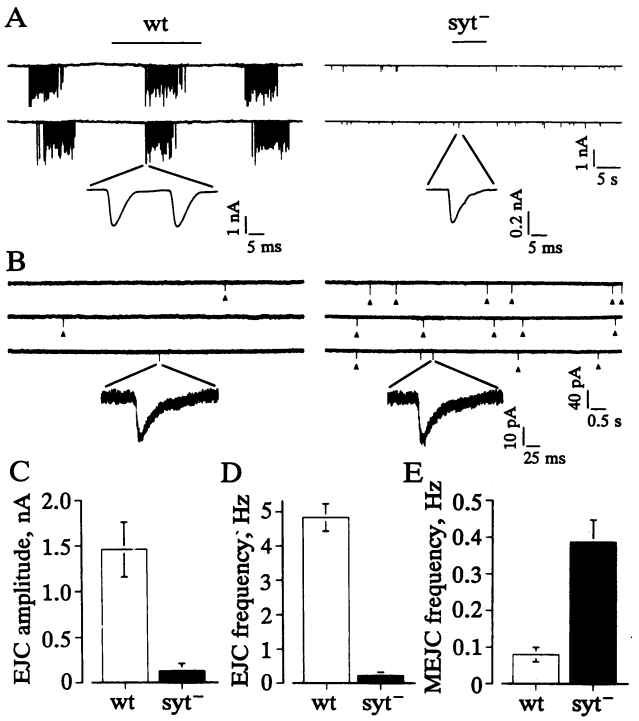

FIG. 2. Synaptotagmin-deficient neuromuscular synapses show dramatically impaired transmission. (*A*) In wt *Drosophila* at hatching (22 hr AEL), neuromuscular transmission occurs as periodic bursts of EJCs with peak amplitudes of several nanoamps. Each burst of EJCs underlies a muscle contraction in the larva's normal locomotory movement. In synaptotagmin null mutants (*syt*⁻) at 22 hr AEL, patterned synaptic transmission is abolished. Residual activity is present as a very low frequency of EJCs with peak amplitudes of a few hundred picoamperes. The time course of wt and mutant EJCs is similar. (*B*) In wt NMJs at hatching (22 hr AEL), spontaneous vesicle fusion in the presynaptic terminal results in a very low frequency of miniature EJCs (MEJCs; arrowheads) in the muscle. In *syt*⁻ NMJs at 22 hr AEL, the frequency of MEJCs is greatly increased relative to wt. The amplitude and time course of wt and mutant MEJCs are similar. (*C*) Mean EJC amplitudes are reduced an order of magnitude in *syt*⁻ synapses relative to wt. (*D*) EJC frequency is reduced more than an order of magnitude in *syt*⁻ synapses relative to wt. (*E*) Spontaneous MEJCs are increased 4- to 5-fold in *syt*⁻ synapses relative to wt. Measurements of endogenous EJCs were made in an intact neuromusculature in 1.8 mM external Ca^{2+}; measurements of MEJCs were made with the motor nerve cut in 0 mM Ca^{2+}. After recording, the genotype was confirmed with an anti-synaptotagmin antibody (Fig. 1). Each measurement represents the mean ± SEM from at least 10 animals of each genotype.

tions by which the larva moves. In *syt*⁻ mutants, this periodic bursting activity at the larval NMJ is abolished. Residual synaptic activity is present as apparently unpatterned currents decreased by an order of magnitude in both frequency and mean amplitude (Fig. 2 *A*, *C*, and *D*). The time course of individual currents is similar to that of wt. This degree of synaptic transmission is sufficient to allow limited mobility so that embryos lacking synaptotagmin can sometimes hatch, but the larvae invariably die soon afterwards.

In addition to evoked responses, spontaneous miniature currents can be recorded at these synapses in standard saline conditions and when evoked release has been blocked by severing the nerve or by using tetrodotoxin or Ca^{2+}-free saline. Surprisingly, at both wt and *syt*⁻ synapses, the time course of the miniature currents is slower than evoked currents by a factor of ≈5 (Fig. 2 *A* and *B*). Though we do not understand this phenomenon, it might arise from an action potential-dependent change in the activity of the presynaptic glutamate uptake system.

While evoked synaptic transmission is suppressed, the frequency of spontaneous miniature currents is increased 5-fold in *syt*⁻ mutants relative to wt (Fig. 2 *B* and *E*). The characteristics of individual miniature currents are unchanged in the mutants; mean amplitude and duration of miniature currents are similar to those in wt (Fig. 2*B*). Thus, the removal of synaptotagmin decreases evoked synaptic vesicle release but increases spontaneous vesicle release.

The physiological defects in *syt*⁻ mutants do not appear to result from morphological abnormalities. The morphology of the NMJ on muscles 6 and 7 was examined with anti-fasciclin II antibody (20), which reveals both the synaptic branches and boutons (sites of transmitter release). The number of boutons, the number and length of synaptic branches, and the total size of the synapse are similar in *syt*⁻ and normal NMJs (data not shown; ref. 18). Thus, the defects in synaptic transmission in the mutant are likely to reflect functional disruption rather than the loss of morphological structures.

In principle, defects in neurotransmission may reflect failure in either postsynaptic or presynaptic mechanisms. However, in *syt*⁻ mutants, the postsynaptic muscle membrane responds to iontophoretically applied transmitter (L-glutamate) in a manner indistinguishable from that of wt (data not shown; ref. 18). The muscle's glutamate transmitter receptors are present at normal density and clustered at the postsynaptic site to form a normal receptor field. Therefore, the observed defects in endogenous transmission must be due to defects in the presynaptic signaling pathway.

A decrease in evoked release could arise from an abnormality in the Ca^{2+}-sensing mechanism or in processes upstream or downstream of Ca^{2+} sensing, such as vesicle availability or fusion. We have examined the defect in *syt*⁻ embryos more closely by stimulating the motor nerve and examining the characteristics of transmission over a range of external Ca^{2+} concentrations (Fig. 3). At low Ca^{2+} concentrations in both wt and mutant synapses, the size of the EJC is decreased, and unitary quantal events can be discerned whose amplitude corresponds to that of the miniature EJCs described above (Figs. 3*A* and 4). *syt*⁻ mutants show distinctive defects in Ca^{2+}-dependent vesicle release. The efficiency of vesicle release is dramatically reduced at all Ca^{2+} levels (Fig. 3 *A* and *B*); vesicle release fails completely at lower Ca^{2+} levels (≤0.1 mM), where wt still maintains reliable transmission, and the mean quantal content is reduced at least 1 order of magnitude at all higher Ca^{2+} levels. However, the amount of evoked release is still sensitive to external Ca^{2+} concentration. In Fig. 3*C*, we plot the relationship between mean quantal content, *m*, and external Ca^{2+} concentration; the slope, *n*, of this relationship indicates the cooperativity of Ca^{2+} in binding to its receptor and stimulating vesicle release. We find that the slope of the Ca^{2+} dependence curves is similar in *syt*⁻ mutants and wt (Fig. 3*C*). Thus, the efficiency of Ca^{2+}-dependent vesicle release is dramatically reduced in *syt*⁻ mutants compared with wt, but the Ca^{2+} sensitivity of vesicle release remains similar.

At hatching, wt neuromuscular synapses show consistent vesicle release upon repeated depolarizing stimulation (Fig. 4). In *syt*⁻ mutants, the amount of vesicle release in response to constant stimuli is erratic and unpredictable (Fig. 4). Most depolarizing stimuli (60–70%) fail to elicit any vesicle release, and those that do generate variable release ranging from a single vesicle up to events >10 times the quantal size. Thus, the absence of synaptotagmin lowers the probability of a stimulus causing a secretion event in a manner that resembles the effect of low external Ca^{2+} (Fig. 4*A*) but increases the variability. Interestingly, these transmission characteristics of *syt*⁻ synapses resemble those of newly forming synapses in the embryo prior to mature differentiation (17). The

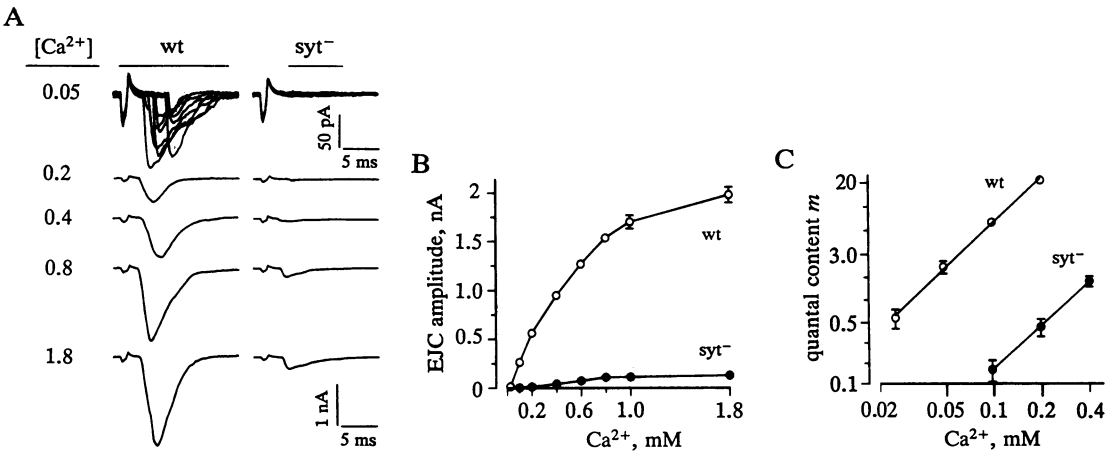

FIG. 3. Ca^{2+}-dependent vesicle release is impaired in synaptotagmin-deficient synapses. (*A*) Synaptic transmission was measured by stimulating the motor nerve in various millimolar concentrations of Ca^{2+} ($[Ca^{2+}]$) and recording the EJC in the voltage-clamped muscle. Here we show representative synaptic current recordings in wt and *syt*$^-$ embryos over a range of external $[Ca^{2+}]$ (shock artifact indicates stimulus time). In low external $[Ca^{2+}]$ (<0.1 mM), evoked vesicle release fails completely in *syt*$^-$ embryos, whereas wt exhibits a low level of vesicle release. Traces in 0.05 mM Ca^{2+} show 10 superimposed responses to a constant stimulus. In higher $[Ca^{2+}]$ (0.1 to 1.8 mM), *syt*$^-$ synapses exhibit limited evoked vesicle release, but the current amplitude is dramatically reduced relative to wt. (*B*) The mean amplitude of the evoked synaptic current is shown over the assayed range of external $[Ca^{2+}]$. wt NMJs show a rapid increase in mean quantal content with increasing $[Ca^{2+}]$ level. *syt*$^-$ NMJs show a dramatic reduction in quantal content at all $[Ca^{2+}]$ levels; mean EJC amplitudes are reduced by a factor of 10 or more at all $[Ca^{2+}]$. (*C*) The mean quantal content *m* is shown relative to $[Ca^{2+}]$ on a log–log plot. The slope (*n*) of this relationship is thought to reflect the cooperativity of Ca^{2+} in binding to its receptor and stimulating vesicle release. In *syt*$^-$ embryos, much higher $[Ca^{2+}]$ levels are required for vesicle release, but the Ca^{2+} dependence of vesicle release is similar in wt ($n = 1.8$) and *syt*$^-$ ($n = 1.7$) embryos. Note that the Ca^{2+} cooperativity relationship is less steep in these immature synapses relative to mature larval synapses ($n \approx 3.6$; ref. 21), suggesting a developmental modification of excitation–release coupling. The genotype of the *syt*$^-$ embryos was confirmed with anti-synaptotagmin staining following recording (Fig. 1). Each point represents the mean ± SEM of at least 500 recordings from 10 embryos of each genotype.

variability in quantal content may reflect either a variability in the number of vesicles available or a variability in the probability that any given vesicle will fuse. Thus, synaptic transmission in *syt*$^-$ synapses is highly unreliable, and the removal of synaptotagmin appears to largely disrupt excitation–vesicle release coupling.

DISCUSSION

Examining synaptic transmission in the absence of synaptotagmin is important for a full understanding of its function. We have demonstrated that synaptotagmin is not required for synaptic transmission at the *Drosophila* NMJ. This conclu-

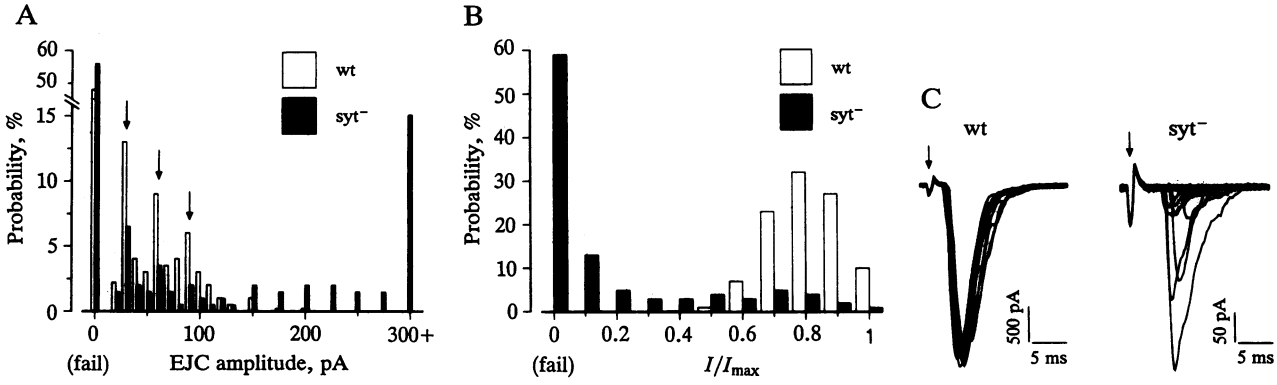

FIG. 4. Null mutations in synaptotagmin largely uncouple the Ca^{2+} influx and vesicle release during synaptic transmission. Single NMJs were stimulated 500 times at 1 Hz, and the amplitude of the evoked synaptic current was recorded from the voltage-clamped muscle. Typical response profiles from wt and *syt*$^-$ embryos are shown. (*A*) Greater variability of release in the mutant is shown by comparing the amplitude distribution of EJCs in *syt*$^-$ and wt embryos in the following conditions that gave approximately equal numbers of failures (fail): 1.8 mM (*syt*$^-$) and 0.02 mM (wt) extracellular Ca^{2+}. For each amplitude, the percent probability of a response is given. The quantal nature of the EJC in both lines can be recognized in the peaks that are observed at amplitudes corresponding to the size of miniature events and their multiples (arrows). The exceptional variability of the *syt*$^-$ embryo responses can be seen in the large number of events that are observed (15%) that are >10 times the quantal size, even in these conditions where most events are failures or single quantal. The bin sizes for the histogram are 10 pA for events smaller than 120 pA and 25 pA for larger events. All events greater than 300 pA are pooled in a single bin (300+). (*B*) Decreased amplitude of *syt*$^-$ embryo events and their greater variability than wt under identical conditions are illustrated by comparison of 500 responses in 1.8 mM Ca^{2+}. Synaptic current amplitude (*I*) is normalized against the maximal response (I_{max}) for each genotype and plotted against the probability (%) of the response. At wt NMJs, constant stimuli produce large consistent EJCs clustered near the maximal response. At *syt*$^-$ NMJs, most stimuli fail to elicit any vesicle release and transmission fails (60–70%). Positive responses show a wide range of synaptic currents elicited from a single vesicle release up to a greatly diminished maximum. (*C*) Representative superimposed current traces from wt and *syt*$^-$ NMJs are shown. Each synapse was repeatedly stimulated under identical conditions in high-Ca^{2+} saline. *syt*$^-$ synapses generate highly variable responses, whereas wt synapses generate consistent responses. Here we show 10 superimposed responses in wt and 25 superimposed responses from a *syt*$^-$ synapse. Latencies varied between preparations, and the difference in latency observed here is not significant. Note the difference in scales. The genotype of the *syt*$^-$ embryos was confirmed with anti-synaptotagmin staining following recording (Fig. 1).

sion is in agreement with previous studies in other systems: in *Drosophila*, and apparently *C. elegans* (13) and PC12 neuroblastoma cells (22), some evoked release persists in the absence of synaptotagmin. Thus, the docking of vesicles at presynaptic release sites and their Ca^{2+}-dependent fusion do not absolutely require synaptotagmin.

Nevertheless, synaptotagmin plays an important regulatory role in excitation–secretion coupling during synaptic transmission *in vivo*. Recent *in vitro* data (23) suggest that synaptotagmin might regulate the cell's constitutive secretion machinery, acting as a clamp to prevent vesicle fusion in the absence of the Ca^{2+} signal. This model predicts that constitutive vesicle fusion should be increased in the absence of synaptotagmin (23). Our observation of an increase in spontaneous miniature EJCs in syt^- mutants is consistent with this model, suggesting that synaptotagmin acts as a negative regulator of secretion *in vivo*.

The observed increase in spontaneous vesicle fusions in syt^- mutants is not in itself sufficient to reduce the vesicle pool to the level required to explain the dramatic reduction in evoked fusion. An active wt synapse releases in excess of 200 vesicles per s (Fig. 2), so it is unlikely that the 0.4 Hz of spontaneous release in the mutant could deplete the synapse of releasable vesicles. Thus, we suggest that synaptotagmin may play additional roles in excitation–secretion coupling. In addition to an inhibitory role, synaptotagmin may also be involved in the docking and fusion of vesicles with the presynaptic membrane. It is possible that synaptotagmin is stabilizing the docked vesicles so that they neither fuse with the membrane nor are lost to the cytosol. Synaptotagmin has been shown to interact with the membrane proteins syntaxin and neurexin (7–10), located at the presynaptic vesicle fusion sites. These interactions might be important in docking or the stabilization of the docked complex so that in synaptotagmin's absence, the fusion-competent pool of vesicles is variably depleted and the fidelity of excitation–secretion coupling is lost.

Synaptotagmin could also play a direct role in the Ca^{2+} activation of vesicle fusion. *In vitro* evidence (5, 6) shows that synaptotagmin can bind Ca^{2+}, resulting in a conformational change that might allow it to serve directly as a Ca^{2+}-sensing mechanism. Our results show that this simple synaptotagmin–Ca^{2+} binding mechanism does not wholly mediate vesicle exocytosis *in vivo* since Ca^{2+}-dependent fusion persists in the absence of synaptotagmin. Moreover, the Ca^{2+} sensitivity of vesicle fusion is unchanged in the absence of synaptotagmin. Therefore, if synaptotagmin is a Ca^{2+} sensor involved in vesicle fusion, it must coexist with an independent Ca^{2+} sensor with similar properties. Alternatively, the Ca^{2+} binding of synaptotagmin might be involved in an earlier stage of the vesicle secretion pathway and not serve directly as a Ca^{2+} sensor mediating the final fusion step.

It is possible that multiple Ca^{2+} sensors exist at the synapse; one candidate is rabphillin (24) whose structure is closely related to that of synaptotagmin. Indeed, inefficient Ca^{2+}-dependent exocytosis occurs in nonneuronal cells [e.g., fibroblasts (25) and muscles (26)] that lack synaptotagmin and so must possess a more general synaptotagmin-independent exocytosis pathway. Therefore, synaptotagmin-mediated transmission in neurons may reflect neuronal specialization of a general exocytosis pathway to allow the extremely rapid, high-frequency vesicle release required at the synapse.

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