

Selective Effects of *neuronal-synaptobrevin* Mutations on Transmitter Release Evoked by Sustained Versus Transient Ca^{2+} Increases and by cAMP

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Synaptobrevin is a key constituent of the synaptic vesicle membrane. The *neuronal-synaptobrevin* (*n-syb*) gene in *Drosophila* is essential for nerve-evoked synaptic currents, but miniature excitatory synaptic currents (mESCs) remain even in the complete absence of this gene. To further characterize the defect in these mutants, we have examined conditions that stimulate secretion. Despite the inability of an action potential to trigger fusion, high K^+ saline could increase the frequency of mESCs 4- to 17-fold in a Ca^{2+} -dependent manner, and the rate of fusion approached 25% of that seen in wild-type synapses under the same conditions. Similarly, the mESC frequency in *n-syb* null mutants could be increased by a Ca^{2+} ionophore, A23187, and by black widow spider venom. Thus, the ability of the vesicles to fuse in response to sustained increases in

cytosolic Ca^{2+} persisted in the absence of this protein. Tetanic stimulation could also increase the frequency of mESCs, particularly toward the end of a train and after the train of stimuli. In contrast, these mutants did not respond to an elevation of cAMP induced by an activator of adenylyl cyclase, forskolin, or a membrane-permeable analog of cAMP, dibutyryl cAMP, which in wild-type synapses causes a marked increase in the mESC frequency even in the absence of external Ca^{2+} . These results are discussed in the context of models that invoke a special role for *n-syb* in coupling fusion to the transient, local changes in Ca^{2+} and an as yet unidentified target of cAMP.

Key words: neuronal-synaptobrevin; miniature synaptic current; high potassium stimulation; tetanic stimulation; cAMP; forskolin; neuromuscular junction; *Drosophila*

The specialization of the presynaptic nerve terminal for fast exocytosis has been widely investigated. It is postulated that molecules on the synaptic vesicle membrane specifically bind to the receptors on the presynaptic membrane and form a docked state. After arrival of an action potential at the presynaptic nerve terminal and the resulting entry of Ca^{2+} through voltage-gated Ca^{2+} channels, the local concentration of Ca^{2+} rises abruptly to a submillimolar level (Simon and Llínas, 1985; Roberts et al., 1990; Adler et al., 1991). This high concentration of Ca^{2+} is detected by a low-affinity Ca^{2+} sensor on the vesicular membrane that initiates rapid vesicle fusion resulting in synchronous transmitter release. On the other hand, spontaneous fusions of synaptic vesicles at the resting state generate miniature synaptic potentials. The frequency of these events changes when the cytosolic Ca^{2+} concentration is altered in the micromolar range (Delaney and Tank, 1994). Therefore, requirements for these two types of transmitter release, namely, action potential-triggered synaptic currents and miniature excitatory synaptic currents (mESCs), may be different.

Synaptobrevin is a major protein of synaptic vesicle membranes and the target of several clostridial neurotoxins (Schiavo et

al., 1992). It is thought to be a crucial determinant of transmitter release and may contribute to membrane fusion or assist in tethering vesicles to appropriate target membranes (Söllner et al., 1993a,b). *Neuronal synaptobrevin* (*n-syb*) is the predominant synaptobrevin gene in *Drosophila* (DiAntonio et al., 1993). At the fly neuromuscular junction, cleavage of this protein by expression of a tetanus toxin transgene removed nerve-evoked synaptic transmission, but mESCs remained (Sweeney et al., 1995). Deitcher et al. (1998) confirmed these findings with a genetic deletion of the *n-syb* locus. These findings raised questions concerning the nature of the residual release of transmitter. Do the vesicles lacking *n-syb* protein retain the ability to be regulated by Ca^{2+} ? Can the absence of a response to an action potential be attributed to a lack of function of the voltage-gated Ca^{2+} channels? In the present study, we have stimulated the fusion of synaptic vesicles by several treatments that produce sustained changes in cytosolic Ca^{2+} and found that the loss of this major vesicular protein produces a specific deficit in the ability of the vesicles to respond to fast, transient increases in cytosolic Ca^{2+} .

At many synapses, cAMP can also facilitate synaptic transmission and is presumed to play a key role in synaptic plasticity (Dixon and Atwood, 1989; Salin et al., 1996; Chen and Regehr, 1997). At the *Drosophila* neuromuscular junction, cAMP has been shown to facilitate synaptic transmission (Zhong and Wu, 1991) and also to increase the frequency of spontaneous transmitter release (Zhang et al., 1999a,b). How does cAMP interact with the two types of transmitter release? We report here that *n-syb* mutants completely lack the response to agents that increase cAMP. It, therefore, appears that *n-syb* will be essential for

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facilitation of transmitter release by cAMP and that cAMP cannot influence the tonic *n-syb*-independent pathway.

MATERIALS AND METHODS

Fly stocks. A detailed genetic and molecular description of the mutants used in this study has been presented in Deitcher et al. (1998). Briefly, the allele *n-syb*^{ΔF33B} contains a deletion encompassing most of the coding region and represents a null allele. We also used *n-syb*^{F33-R}, which is homozygous lethal and closely resembles a null allele but could produce some *n-syb* protein. As a control, the parental strain, line 34, was used in the majority of experiments, although Canton S was also used in some early experiments. Each stock was maintained in a *y, w* background and balanced with a *TM6, y+ Ubx* chromosome. Homozygous mutant embryos were identified by yellow mouth hooks. Mutant embryos were used at 21–24 hr after egg laying. Because by this time line 34 or Canton S larvae were already hatched, those newly hatched larvae were used for controls.

Electrophysiology. The dissecting and procedures for recording synaptic currents were described elsewhere (Kidokoro and Nishikawa, 1994; Nishikawa and Kidokoro, 1995). Dissection was done in Ca²⁺-free, Mg²⁺ saline. The ventral ganglion was kept intact. After dissection, the preparation was treated for 1–3 min with 1 mg/ml collagenase (type IV; Sigma, St. Louis, MO) in 0.1 mM Ca²⁺ saline. To measure the frequency of spontaneous synaptic currents in high K⁺ saline, we kept the preparation in Ca²⁺-free solution for 3 min before changing to the solution containing a desired Ca²⁺ concentration.

Mainly longitudinal muscles 6, 7, and 4 were voltage-clamped at –60 mV, and currents were filtered at 5 kHz. In the majority of experiments, the mESC frequency was visually counted for 5 min on a CRT screen. To avoid missing events during fly-back time of CRT sweeps, we simultaneously used a paper recorder (Nihon-Kohden, Tokyo, Japan), but when the frequency was high, a computer was used to count synaptic currents for 1 min. Spontaneous synaptic currents with a slow time course were mixed with fast ones because of electrical coupling of muscle cells with those in neighbor segments (Kidokoro and Nishikawa, 1994; Gho, 1994; Ueda and Kidokoro, 1996). In this study only synaptic currents with a fast time course were counted. Each synaptic current was observed on the CRT screen, and mESCs with slow time courses (>2 msec in the rise time) were rejected from counting.

In both *n-syb* mutant embryos and line 34 larvae, stretching nerve terminals in higher concentrations of external Ca²⁺ (≥0.5 mM) caused high frequencies of synaptic currents even in the presence of tetrodotoxin (TTX). In this study we tried to avoid stretching preparations during dissection and did not record from cells that were distorted because of the contraction of neighboring muscle cells. Even with these precautions we did observe cells with very high frequencies of mESCs in *n-syb* mutant embryos as well as controls. However, these anomalously high frequency fibers were excluded from the data.

All electrophysiology experiments were performed at room temperature (22–26°C).

Solutions. The ionic composition of solutions used in the experiments are as follows (in mM): normal external saline: NaCl, 140; KCl, 2; MgCl₂, 5.5; CaCl₂, 0.5; and HEPES-NaOH, 5, pH 7.1; Ca²⁺-free external solution: NaCl, 140; KCl, 2; MgCl₂, 6; and HEPES-NaOH, 5, pH 7.1; and high K⁺ external saline: NaCl, 122; KCl, 20; MgCl₂, 5.5; CaCl₂, 0.5, and HEPES-NaOH, 5, pH 7.1. High K⁺ external salines with various concentrations of Ca²⁺ were prepared by replacing MgCl₂ with CaCl₂. The ionic composition of the internal solution was (in mM): CsCl, 158; ATP, 2; EGTA, 5; and HEPES-NaOH, 10, pH 7.1.

Biochemicals and toxins. TTX and Ca²⁺ ionophore A23187 were purchased from Sigma. A23187 was dissolved in DMSO at 5 mM and used at 10 μM in the external solutions with various Ca²⁺ concentrations. A synthetic toxin, PLTX-II, was purchased from Peptide Institute (Osaka, Japan).

Black widow spider venom (BWSV) was a gift from Drs. Joy A. Umbach and Cameron B. Gundersen at University of California at Los Angeles. A gland was dissolved in 100 μl of Na/Tris-saline (150 mM NaCl and 20 mM Tris-HCl, pH 7.5) and stored at –70°C. Shortly before use, the stock solution was thawed and diluted into the external solution (1/60) with 0.5 or 2 mM Ca²⁺ for *n-syb* mutant embryos and 0.2 or 0.5 mM Ca²⁺ for line 34 larvae. With perfusion, ~0.7 ml of the toxin-containing solution was introduced into the bath with a volume of 1.2–1.5 ml. Thus, there was another approximately threefold dilution of the toxin in this process. The final concentration was ~0.056 gland/ml.

Forskolin was dissolved in 100% ethanol at the concentration of 10

mM. Forskolin-containing Ca²⁺-free solution (0.5 ml) of two times a desired concentration was added to the bath solution of 0.5 ml and stirred so that the final concentration of forskolin became the desired concentration. In this experimental condition, when the highest concentration (500 μM) of forskolin was used, the ethanol concentration became as high as 5%, but 5% ethanol dissolved in Ca²⁺-free external solution by itself did not have any effect on the mESC frequency. Dibutyryl cAMP is water soluble and used at 1 mM. For application of dibutyryl cAMP, the volume of bath solution was reduced to 0.15 ml before infusion of the drug containing solution with a volume of 1.1 ml. Consequently, there was a slight dilution of dibutyryl cAMP to a final concentration of 0.86 mM. The perfusion rate was 0.73 ml/min, and the pump was stopped after infusion of dibutyryl cAMP. Forskolin and dibutyryl cAMP were purchased from Wako Chemicals (Osaka, Japan).

RESULTS

Movements in *n-syb* null embryos suggest that some neuromuscular communication persists

Our previous studies (Deitcher et al., 1998) have indicated that spontaneous mESCs are present in *n-syb* null mutants, but that action potentials in the nerve could not evoke a response in the muscle. Nerves were stimulated at 0.3 Hz in 2 mM Ca²⁺ saline and in the presence of 2 mM 4-AP to prolong Ca²⁺ entry into the terminal, and recordings from the postsynaptic muscle fiber were examined for a response in the 10 msec interval after the stimulus. In control lines, such a protocol consistently would have produced large, multiquantal responses to each action potential. Yet in the *n-syb* mutants, every stimulus failed. These results were in accord with similar low-frequency stimulation experiments in tetanus toxin-expressing transformants (Broadie et al., 1995; Sweeney et al., 1995); yet, observations of the behavior of the embryos suggested to us that some nerve-evoked contractions might be occurring in the muscles. In their gross morphological features, *n-syb* null mutant embryos resembled those of control larvae (line 34) of a similar age. At 21–24 hr after egg laying, the mutant embryos had aerated tracheae but did not move spontaneously within the vitelline membrane; yet, when the membrane was torn by a sharp needle, they invariably displayed wiggly movements. For several minutes, segmental muscles at various positions contracted sporadically until the embryos eventually became placid. When embryos were prepared for electrophysiological recordings by dissection in the presence of 1 or 2 mM Ca²⁺ in the external saline, spontaneous and uncoordinated contractions of the muscles were again observed. If these contractions and those observed after devitellinization were entirely myogenic, they should be insensitive to TTX. We observed, however, the 3 μM TTX caused most of the movements to disappear, suggesting a neural origin. These observations and a more general interest in the nature of the *n-syb*-independent pathway to transmitter release led us to examine the neuromuscular junctions more closely in these mutants. As described below, we have found that sustained Ca²⁺ influx into the nerve terminal, as a result of K⁺ depolarization or tetanic stimuli, can indeed stimulate the release of transmitter.

The frequency of mESCs in *n-syb* null mutants can be modulated

In all cells examined in *n-syb*^{ΔF33B} and *n-syb*^{F33-R} embryos, spontaneous synaptic currents were observed at low frequencies in normal saline (~1/min) (Deitcher et al., 1998). The frequency was higher in high K⁺ saline in the presence of external Ca²⁺. This observation suggests that Ca²⁺ ions do enter the terminal in a voltage-dependent manner in mutant embryos and are detected by the Ca²⁺ sensor for an acceleration of spontaneous release of transmitter. These spontaneous synaptic currents were mESCs

because they remained even in the presence of 3 μM TTX. Because the mESC frequency was low in normal saline, it was difficult to collect enough events for a detailed analysis. Therefore, we used high K^+ external solution to facilitate the occurrence of mESCs and examined the effect of external Ca^{2+} on their frequency.

The mESCs were studied in the presence of 3 μM TTX and 20 mM KCl in the external solution, and the concentration of external Ca^{2+} was varied between 0 and 2 mM. Spontaneous synaptic currents were observed in low frequencies even in the absence of external Ca^{2+} in mutant embryos. The mean frequency was $1.6 \pm 0.7/\text{min}$ (mean \pm SD, $n = 10$; data will be expressed in this format throughout the text unless otherwise specified) in *n-syb* $^{\Delta\text{F33B}}$ embryos and $1.5 \pm 1.5/\text{min}$ ($n = 14$) in *n-syb* $^{\text{F33-R}}$ embryos. These frequencies were slightly smaller than that in line 34 larvae ($3.3 \pm 2.8/\text{min}$; $n = 10$). This observation at zero external Ca^{2+} is in accord with that reported for tetanus toxin-expressing transformants (Broadie et al., 1995).

As the external Ca^{2+} concentration was increased, the frequency was elevated. With 2 mM Ca^{2+} in the external solution, the mean frequency was $13.5 \pm 13.0/\text{min}$ ($n = 16$) in *n-syb* $^{\Delta\text{F33B}}$ embryos and $10.5 \pm 11.8/\text{min}$ ($n = 15$) in *n-syb* $^{\text{F33-R}}$ embryos, whereas it was $68.2 \pm 85.9/\text{min}$ ($n = 8$) in line 34 larvae. In Figure 1, the mean frequency was plotted against the external Ca^{2+} concentration. The curves for mutant embryos (*n-syb* $^{\Delta\text{F33B}}$ embryos, dotted line with squares and *n-syb* $^{\text{F33-R}}$ embryos, broken line with triangles) were always below that for line 34 larvae (solid line with circles). This could be either caused by less Ca^{2+} entering the presynaptic terminal or a less efficient vesicle fusion machinery for spontaneous release in *n-syb* mutant embryos. We favor the latter possibility based on observations with a Ca^{2+} ionophore and black widow spider venom as described below.

The increase in mESC frequency in the high K^+ solution was blocked by Cd^{2+} or PLTX-II in *n-syb* mutant embryos

To test whether the increase in the mESC frequency with external Ca^{2+} in high K^+ saline is caused by activation of voltage-gated Ca^{2+} channels, we examined the effect of 1 mM Cd^{2+} . When 1 mM Cd^{2+} was included in the external solutions together with 2 mM Ca^{2+} , the mean frequency was reduced to the level seen in 0 Ca^{2+} (Fig. 2). Because Cd^{2+} is known to be an effective blocker of voltage-gated Ca^{2+} channels, this result suggests that the effect of external Ca^{2+} on the mESC frequency was caused by Ca^{2+} influx through voltage-gated Ca^{2+} channels.

Furthermore, a spider toxin, PLTX-II, is known to inhibit Ca^{2+} currents in *Drosophila* neurons (Leung et al., 1989) and block neuromuscular transmission at the *Drosophila* neuromuscular junction (Branton et al., 1987). Therefore, it has been postulated that PLTX-II blocks voltage-gated Ca^{2+} channels at the presynaptic terminals and thereby eliminates nerve-evoked synaptic currents. We tested the effect of PLTX-II on the increase of mESC frequency in high K^+ saline in *n-syb* mutant embryos. As shown in Figure 2 (horizontally striped column), in *n-syb* $^{\Delta\text{F33B}}$ embryos the increase of mESC frequency in the high K^+ solution in the presence of 2 mM Ca^{2+} was completely blocked by 50 nM PLTX-II.

Similar effects were also found in line 34 larvae in the presence of 1 mM Ca^{2+} . Cd^{2+} (0.5 mM) reduced the mESC frequency from $18.4 \pm 4.6/\text{min}$ ($n = 9$) to $3.8 \pm 1.7/\text{min}$ ($n = 8$). The latter frequency was similar to the level observed in 0 mM Ca^{2+} ($3.3 \pm 0.9/\text{min}$; $n = 10$). PLTX-II (50 nM) also reduced the increase of

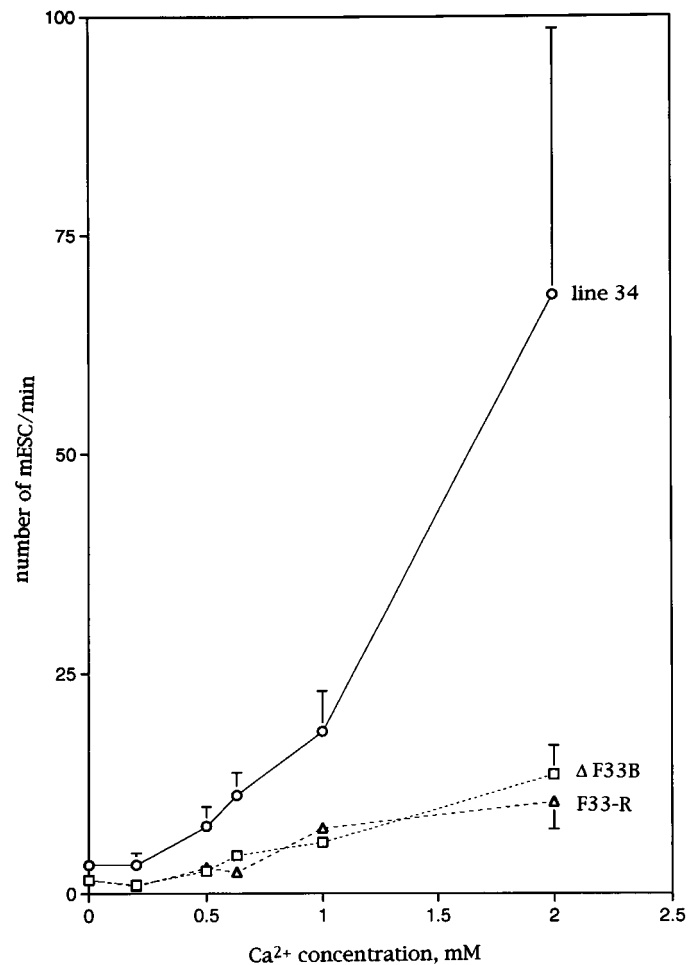


Figure 1. The relation between the frequency of mESCs and external Ca^{2+} concentration in the 20 mM K^+ solution in the presence of 3 μM TTX. The frequency was plotted against external Ca^{2+} concentration. Open circles are for line 34 larvae, squares are for *n-syb* $^{\Delta\text{F33B}}$, and triangles are for *n-syb* $^{\text{F33-R}}$. Error bars indicate SEM. Neighboring data points were connected by straight lines.

mESC frequency in 0.5 mM Ca^{2+} [$7.7 \pm 2.2/\text{min}$ ($n = 13$) to $1.7 \pm 0.2/\text{min}$ ($n = 5$)]. These results suggest that Cd^{2+} - and PLTX-II-blockable Ca^{2+} channels are operating in *n-syb* mutant embryos.

Ca^{2+} ionophore, A23187, also increased the mESC frequency in the presence of external Ca^{2+} in *n-syb* $^{\Delta\text{F33B}}$ embryos

The lower frequency of mESCs in *n-syb* mutant embryos relative to line 34 larvae at a given concentration of external Ca^{2+} could either be caused by less Ca^{2+} entry or reduced efficiency of the fusion mechanism. Syntaxin is known to reduce currents through N-type Ca^{2+} channels by shifting the steady-state inactivation curve to the left along the voltage axis (Bezprozvanny et al., 1995). Therefore, it is plausible that neuronal synaptobrevin, which binds to syntaxin together with *N*-ethylmaleimide-sensitive factor and soluble *N*-ethylmaleimide-sensitive factor attached protein (Söllner et al., 1993a,b), might indirectly affect Ca^{2+} channels and that the lack of neuronal synaptobrevin in *n-syb* mutant embryos might reduce Ca^{2+} influx associated with an action potential. To distinguish these two alternatives, it is necessary to increase the cytoplasmic Ca^{2+} concentration in the

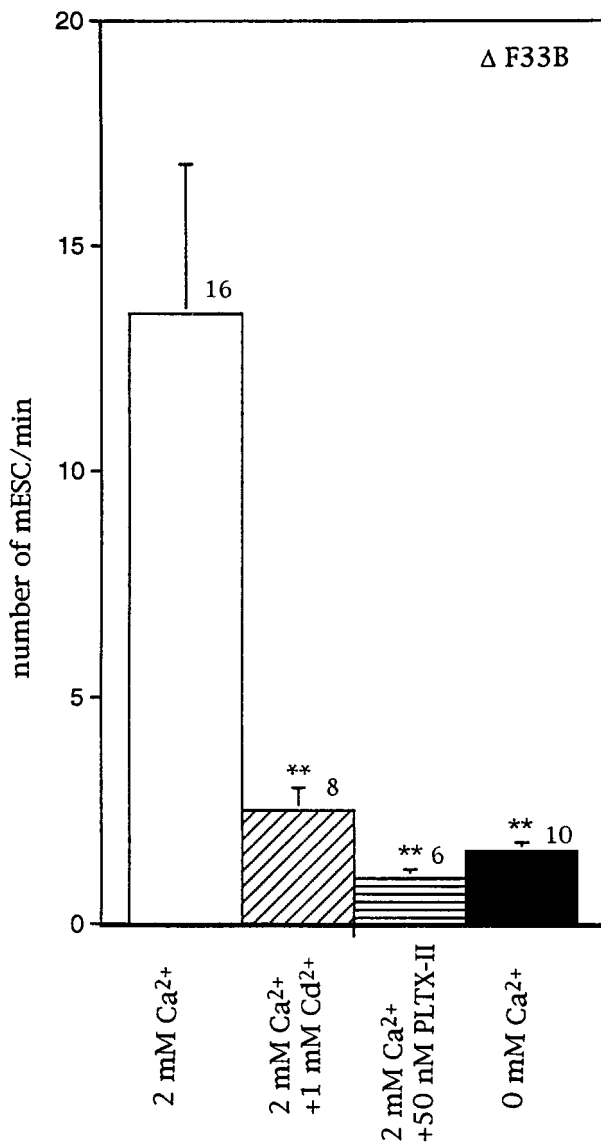


Figure 2. Effects of Ca²⁺ channel blockers on the mESC frequency in *n-syb*^{ΔF33B}. The frequency of mESCs were counted in the 20 mM K⁺ solution in the presence of 3 μM TTX. Conditions for each experiment are written below the abscissa. Error bars indicate SEM. Two asterisks on each column indicate statistical difference at *p* = 0.01 from control (blank column).

presynaptic terminal by other routes than voltage-gated Ca²⁺ channels.

We used a Ca²⁺ ionophore, A23187, to increase Ca²⁺ concentrations in the nerve terminal independently from voltage-gated Ca²⁺ channels. In *n-syb*^{ΔF33B} embryos treated with A23187, the frequency of mESCs increased in the presence of 0.5 mM Ca²⁺ compared with that in 0 mM Ca²⁺ (*p* < 0.01) (Fig. 3, circles). With 0.2 mM Ca²⁺, the effect was intermediate. A23187 itself did not increase the mESC frequency independently of Ca²⁺ entry: in 0 Ca²⁺ the frequency was 1.3 ± 0.9/min (*n* = 10) with the ionophore and 1.1 ± 0.9 (*n* = 5) without. The extent of the increase in *n-syb*^{ΔF33B} embryos was less than that in line 34 (Fig. 3, squares). Because we have no reason to think the ionophore behavior of A23187 would be affected by the mutation, we suggest that the vesicle fusion machinery in *n-syb*

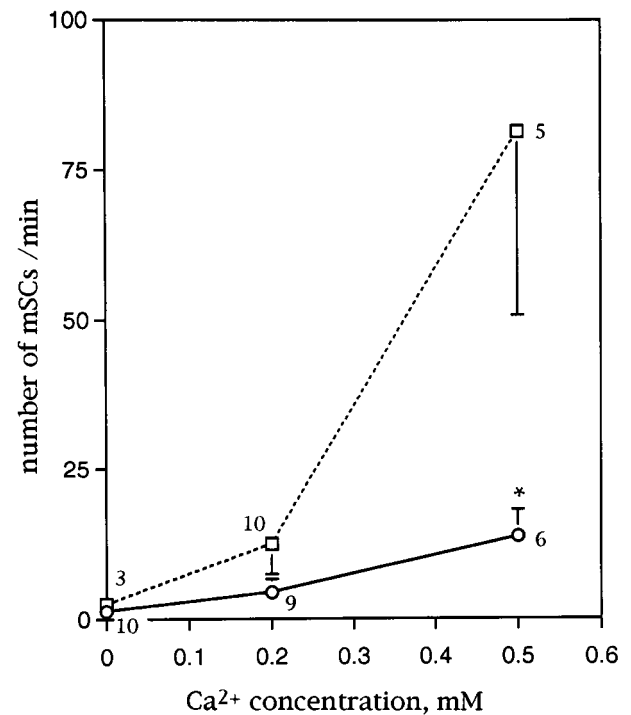


Figure 3. Effects of Ca²⁺ ionophore A23187. The number of mESCs per minute is plotted against the external Ca²⁺ concentration. Squares are for line 34 larvae, and open circles are for *n-syb*^{ΔF33B} embryos. Error bars indicate SEM, and numbers are the number of cells examined. An asterisk indicates statistical difference between line 34 and *n-syb*^{ΔF33B} at *p* = 0.05.

mutant embryos is less effective after elevation of cytosolic Ca²⁺ than that in line 34 larvae, as when high K⁺ was used to elevate intracellular Ca²⁺, the rate of release in *n-syb*^{ΔF33B} was ~20% of wild-type control.

BWSV also increased the mESC frequency in *n-syb*^{ΔF33B} embryos

BWSV increases the internal Ca²⁺ concentration and causes massive release of transmitter (Meldolesi et al., 1986). Considering the results described above with high K⁺ saline and Ca²⁺ ionophore, we would expect that BWSV also increases the mESC frequency in *n-syb* mutant embryos, although the mechanism of action of BWSV on insect synapses is not well characterized. Contrary to this expectation, Broadie et al. (1995) reported that BWSV did not increase the mESC frequency in a *Drosophila* transformant in which neuronal synaptobrevin was cleaved by tetanus toxin. To resolve this discrepancy we tested BWSV in *n-syb*^{ΔF33B} embryos. BWSV was added in the bath by perfusion while synaptic current recordings were made. All solutions used in these experiments contained 3 μM TTX to avoid an indirect effect of BWSV through the ventral ganglion. After ~0.7 ml of the solution containing BWSV was introduced in the bath, perfusion was stopped while the mESC frequency was continuously monitored. The effect became suddenly evident 3–5 min after addition of the toxin to the bath and slowly declined within ~10 min, even in the presence of the toxin (Fig. 4), but the extent of increase, measured at the peak, was less in *n-syb* mutant embryos (circles) compared with line 34 larvae (squares). As shown in Table 1, BWSV significantly increased the mESC frequency in *n-syb*^{ΔF33B} embryos with 0.5 and 2 mM Ca²⁺ in the external solution. The effect was more prominent in 2 mM than in 0.5 mM external Ca²⁺.

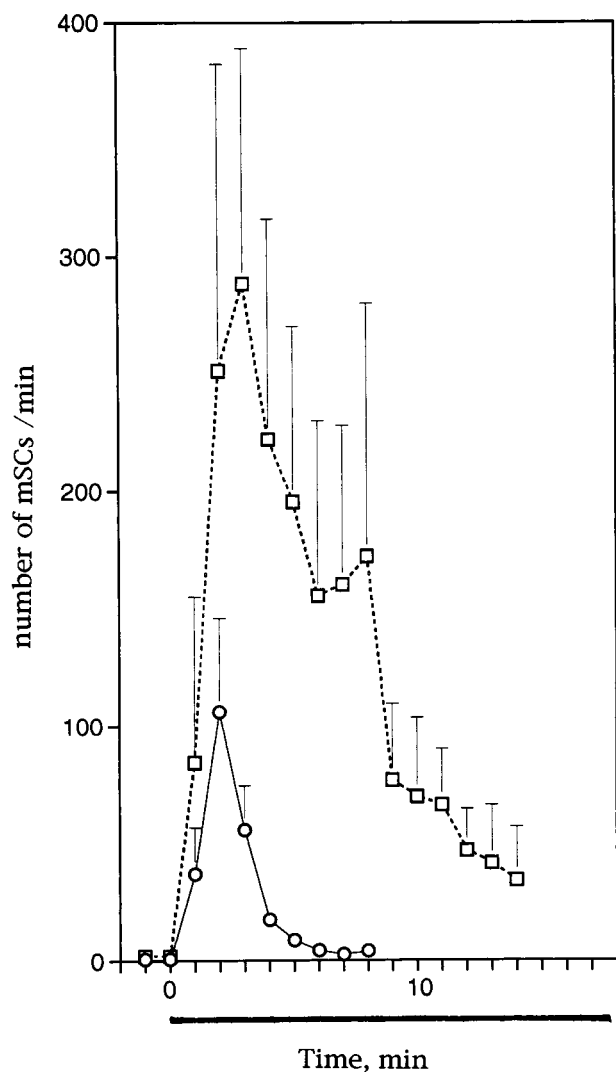


Figure 4. Effects of BWSV on the frequency of mESCs. BWSV was applied during the period shown by a horizontal dark line at the bottom. Squares are for line 34 larvae (4 preparations), and open circles are for *n-syb*^{ΔF33B} embryos (3 preparations). Error bars indicate SEM. The frequency of mESCs was measured in the external solution containing 0.5 mM Ca²⁺ and 3 μM TTX.

While in line 34 larvae the effect was vigorous in 0.5 mM Ca²⁺, and a significant increase in the mESC frequency was observed even in 0.2 mM Ca²⁺.

Thus, BWSV did increase the mESC frequency in *n-syb* mutant embryos, and the extent of the effect was dependent on the external Ca²⁺ concentration. BWSV was, however, less effective in *n-syb* mutant embryos than in line 34 larvae: the peak rate of release was ~1/3 that of the control. This result is in accord with reports on the effect of BWSV on tetanus toxin-treated preparations (Janicki and Habermann, 1983; Dreyer et al., 1987; Gansel et al., 1987). However, it is not clear at this moment why the effect of BWSV was not observed in the tetanus toxin-expressing transformants by Brodie et al. (1995).

Tetanic stimulation induced release of transmitter in *n-syb* mutant embryos

So far we have shown that the mESCs in *n-syb* mutant embryos respond to an elevation of internal Ca²⁺. However, nerve stim-

Table 1. Effects of black widow spider venom on the mESC frequency

External Ca ²⁺ concentration (mM)	<i>n-syb</i> ^{ΔF33B}		Line 34	
	BWSV (-)	BWSV (+)	BWSV (-)	BWSV (+)
0.2			2.3 ± 0.6 <i>n</i> =3	58 ± 21 <i>n</i> =3
0.5	0.7 ± 0.2 <i>n</i> =4	162 ± 59 <i>n</i> =3	2.0 ± 0.7 <i>n</i> =5	553 ± 225 <i>n</i> =4
2.0	0.8 ± 0.1 <i>n</i> =6	381 ± 38 <i>n</i> =3		

Numbers are the average number of mESCs per minute ± the SEM and the number of cells examined (*n*). BWSV (-) indicates measurements in the absence of black widow spider venom, and BWSV (+) shows those in the presence of the venom.

ulation at the low frequency does not evoke synaptic currents in the mutant embryos (Deitcher et al., 1998). Is it possible that nerve stimulation does not elevate internal Ca²⁺ in mutants? To test this possibility, we stimulated the nerve repetitively at 10 Hz and examined the frequency of mESCs during and after the tetanus.

Spontaneous synaptic currents in *n-syb*^{ΔF33B} and *n-syb*^{F33-R} embryos were rare in the 2 mM Ca²⁺ solution with 2 mM K⁺. However, with tetanic stimulation (10–20 pulses at 10 Hz), release of individual quanta of transmitter was observed toward the end of the tetanus and for ~1 sec thereafter (Fig. 5*Aa,Ba*). When the frequency histogram was constructed along the time axis and was aligned to the tetanus, this trend became evident (Fig. 5*Ab,Bb*). Some of these fusions occurred with the right latency for evoked release, namely between 4 and 10 msec after onset of the individual stimuli within the tetanic train (Fig. 5*Aa,Ba*, arrows). This timing suggested that they were indeed coupled to the preceding action potential.

In line 34 larvae with low concentrations of external Ca²⁺ (0.2 mM), individual stimuli did not evoke synaptic currents in the majority of cases, but tetanic stimulation did evoke release of transmitter as shown in Figure 5*Ca*. The frequency histogram shown in Figure 5*Cb* was similar to that in *n-syb* mutant embryos described above except that the synaptic currents that were evoked were synchronized to stimuli more frequently than those in *n-syb* mutant embryos at 2 mM Ca²⁺ (Fig. 5*Ab,Bb*, arrows).

Tetanic stimulation in the absence of Ca²⁺ in the external solution did not evoke synaptic currents in *n-syb* mutant embryos, indicating that Ca²⁺ influx induced by tetanic stimulation is causing the observed release of transmitter. This observation again suggests that voltage-gated Ca²⁺ channels are operating in *n-syb* mutant nerve terminals. In accord with this observation, tetanus toxin treatment of the squid giant synapse markedly reduced synaptic transmission but did not affect Ca²⁺ influx (Hunt et al., 1994).

Because, in addition to mESCs after tetanic stimulation, some of the quanta appeared to have the appropriate latency for having been evoked by the immediately preceding stimulus within each tetanus, we looked at the timing of the mESCs in greater detail (Fig. 6*A*). To distinguish a genuine coupling of stimulus and release from coincidental occurrence of asynchronous events during the 4–10 msec poststimulus interval, we constructed a latency histogram (Fig. 6*Ab*) to describe the interval between the onset of the stimulating pulse and each of the observed synaptic currents. In these histograms it became clear that the earlier bins had more entries than later ones. Thus, stimuli did evoke synaptic

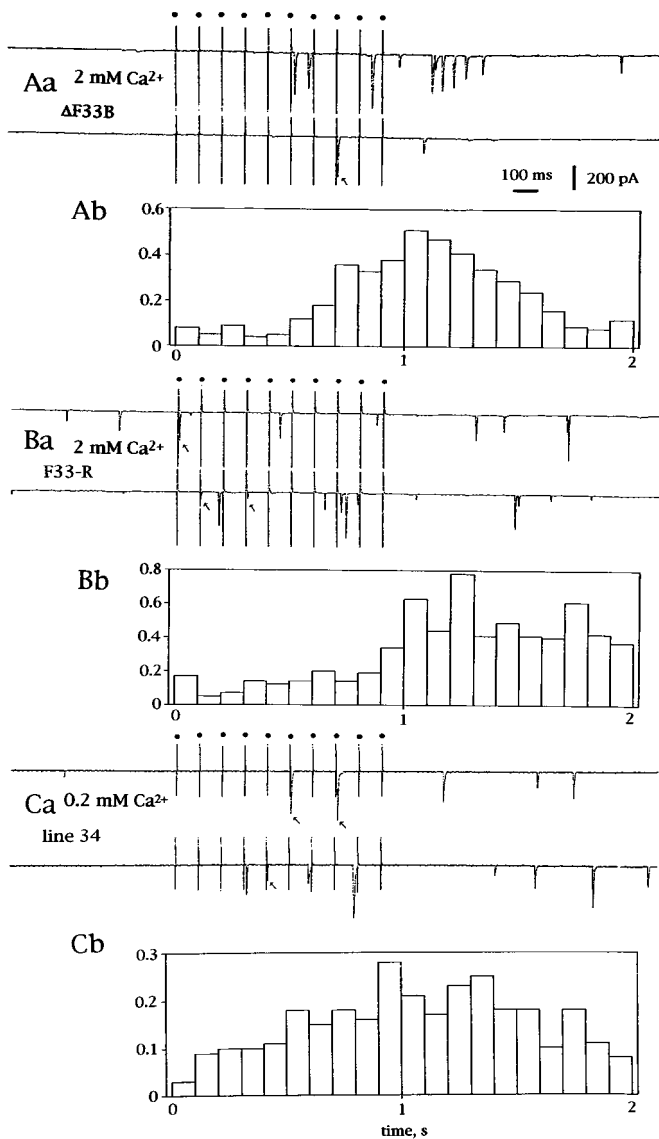


Figure 5. Release of transmitter during and after tetanic stimulation. Ten pulses at 10 Hz were delivered while the muscle cell was voltage-clamped at -60 mV. *Aa*, Current traces on a paper recorder during and after stimulation recorded in an *n-syb* $^{\Delta F33B}$ embryo. The external solution contained 2 mM Ca^{2+} and 4 mM Mg^{2+} . Each stimulus was given at a dot indicated at the top. Downward deflections indicate inward currents. *Ab*, The number of events per 100 msec of tetanic stimulation is plotted on the ordinate. A total of 76 sets of 10 tetanic stimuli were delivered. Timing is aligned to the top traces. *Ba*, Current traces on a paper recorder during and after tetanic stimulation recorded in an *n-syb* $^{F33-R}$ embryo. *Bb*, Number of events per 100 msec of tetanic stimulation. A total of 59 sets of 10 stimuli were delivered. The external solution contained 2 mM Ca^{2+} and 4 mM Mg^{2+} . *Ca*, Current traces on a paper recorder during and after stimulation recorded in a line 34 larva. The external solution contained 0.2 mM Ca^{2+} and 5.8 mM Mg^{2+} . Arrows indicate evoked synchronized synaptic currents. *Cb*, Number of events per 100 msec of tetanic stimulation. A total of 119 sets of 10 tetanic stimuli were delivered.

currents, although the latency was fluctuating, and the synchronous release was extremely rare even in the presence of 2 mM Ca^{2+} . We defined synchronized transmission as synaptic currents that appeared within 10 msec after the onset of stimuli and calculated the percentage of individual stimuli that successfully evoked synchronized transmission. It was $1.5 \pm 1.6\%$ ($n = 7$) for *n-syb* $^{\Delta F33B}$ embryos in 2 mM Ca^{2+} , which was smaller than that of

line 34 larvae in 0.2 mM Ca^{2+} , $13.4 \pm 9\%$ ($n = 5$). Interestingly, the percentage of synchronized transmission for *n-syb* $^{F33-R}$ embryos at 2 mM Ca^{2+} was $9.1 \pm 4.7\%$ ($n = 8$), which is significantly larger than that of *n-syb* $^{\Delta F33B}$ embryos in 2 mM Ca^{2+} ($p < 0.002$, student's two-tailed *t* test). This result suggests that *n-syb* $^{F33-R}$ is not a complete null allele, and some *n-syb* protein being synthesized in these embryos might be making this difference.

In accord with this interpretation, the latency histogram had relatively more entries in the first bin (between 4 and 10 msec) in an *n-syb* $^{F33-R}$ embryo (Fig. 6*Bb*). To document this observation in populations of mutant embryos, we calculated the percentage of events that belonged to the first bin among all events that occurred within 54 msec after stimuli. It was $17.8 \pm 6.5\%$ ($n = 6$) for *n-syb* $^{\Delta F33B}$ embryos, whereas that for *n-syb* $^{F33-R}$ embryos was $31.3 \pm 10.0\%$ ($n = 6$). These values are statistically different ($p < 0.02$). This observation suggests that a role of *n-syb* protein in synaptic transmission is to facilitate fast vesicle fusion after arrival of an action potential in the presynaptic terminal.

cAMP failed to elevate the frequency of mESCs in *n-syb* mutants

At the *Drosophila* neuromuscular junction, metabotropic glutamate receptors cause a tonic increase in the frequency of mESCs by increasing cAMP levels (Zhang et al., 1999a,b). This action appears to be caused by a stimulation of the Ca^{2+} /calmodulin-dependent adenylate cyclase and is responsible for the phenomena of synaptic facilitation and post-tetanic potentiation in this preparation. The means by which cAMP stimulates vesicle fusion is not as yet known, but it occurs in the absence of external Ca^{2+} and therefore cannot entirely consist of the modulation of ion channels in the terminal membrane (Siegelbaum et al., 1982). We have investigated the interaction of cAMP with the two pathways defined by the *n-syb* mutations in the experiments above. If cAMP were acting by raising cytosolic Ca^{2+} levels, we would predict that agents that increase mESC frequency (forskolin and dibutyryl cAMP) would continue to be effective in the mutants: stimulation of the cAMP pathway would resemble the tonic elevation of intracellular Ca^{2+} produced by high K^+ or ionophores. On the other hand, if cAMP acts on a component of the exocytotic apparatus that is unique to the phasic pathway, it would be ineffective in the *n-syb* mutations that lack this pathway.

Experiments with the adenylate cyclase activator forskolin and with dibutyryl cAMP were conducted in Ca^{2+} -free saline to separate the intracellular actions of these agents from any potential actions on Ca^{2+} influx. When applied to a control preparation (line 34), forskolin increased the mESC frequency at relatively high concentrations (a partial dose–response curve is shown in Fig. 7). Forskolin (at a final concentration of 500 μM) increased the mESC frequency 2–7/min (3.8 ± 2.3 /min; $n = 4$) to 6–295/min (133.6 ± 137.1 /min; $n = 4$) (Fig. 8*Aa*). Although the time course was variable, the response was observed in all four cases examined. This result is in accord with the previous report on wild-type larvae (Zhang et al., 1999a,b). In contrast, in *n-syb* $^{\Delta F33B}$ embryos, the mESC frequency was 5.6 ± 1.9 /min ($n = 5$) before application of the drug and was 1.9 ± 1.4 /min ($n = 5$) in 500 μM forskolin for >10 min (Fig. 8*Ab*). In all five cases examined, no response was observed. Similarly, in *n-syb* $^{F33-R}$ embryos, the mESC frequency was 4.3 ± 1.4 /min ($n = 5$) before and 1.0 ± 0.5 /min ($n = 5$) in 500 μM forskolin (Fig. 8*Ac*). Thus, the response to forskolin was completely absent in both *n-syb* mutant embryos.

As an independent means of elevating cAMP levels in the terminals, we used a membrane-permeable analog, dibutyryl

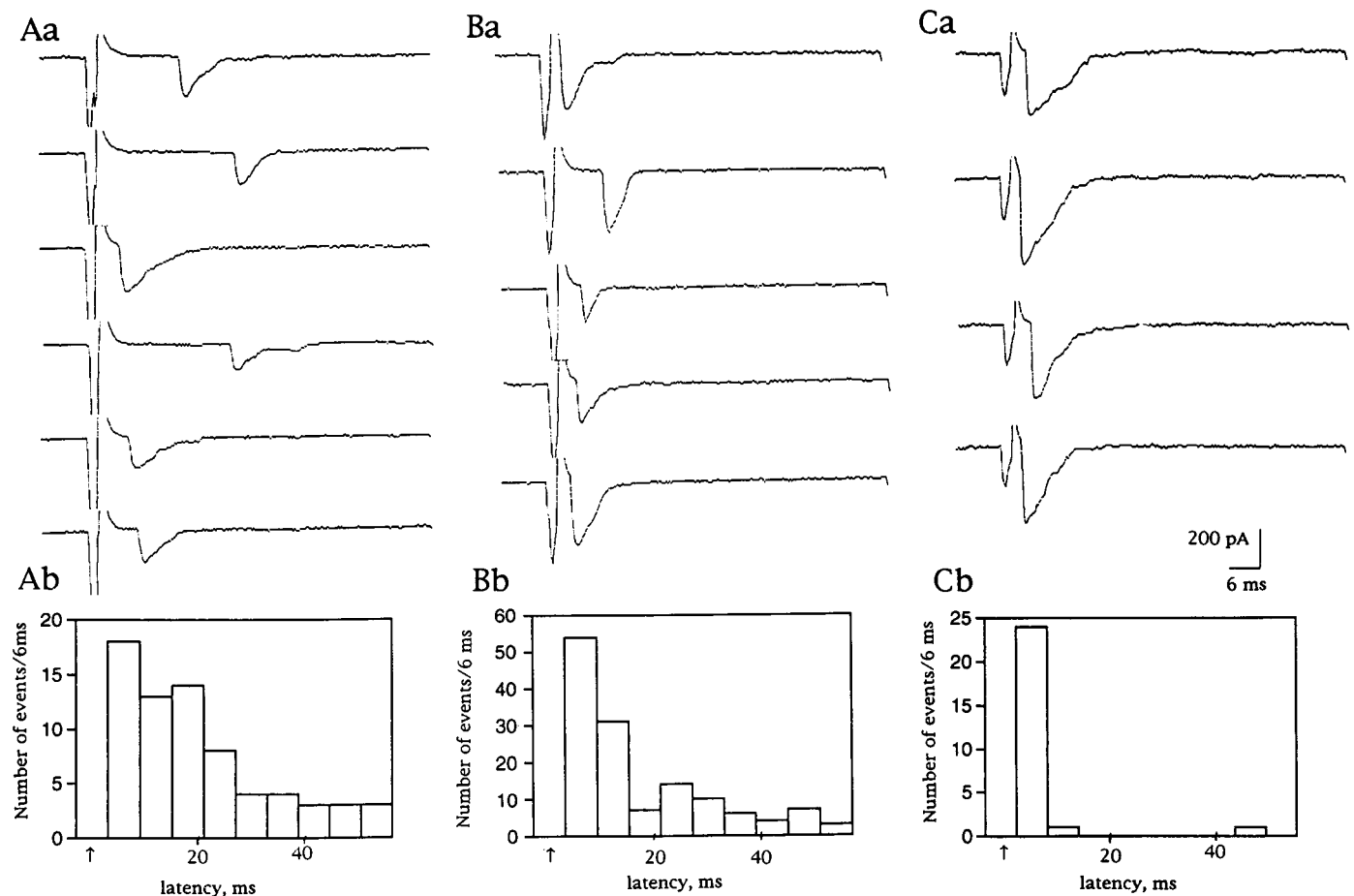


Figure 6. Loosely synchronous release of transmitter in an *n-syb*^{ΔF33B} embryo in 2 mM Ca²⁺ (*Aa*, *Ab*), in an *n-syb*^{F33-R} in 2 mM Ca²⁺ (*Ba*, *Bb*), and synchronous release in a line 34 larva in 0.2 mM Ca²⁺ (*Ca*, *Cb*). *Aa*, Sample traces during tetanic stimulation that had synaptic currents were selected. *Ab*, The latency histogram of synaptic currents occurred after 1841 stimuli during tetani. On the ordinate the cumulative number of events belonging to a bin is plotted. The abscissa is aligned to the above sample traces. Upward arrows indicate the stimulus onset. *Ba*, Selected sample traces during tetanic stimulation. *Bb*, The latency histogram was constructed out of synaptic currents occurred after 792 stimuli during tetanic stimulation. *Ca*, Sample traces that occurred during tetanic stimulation. *Cb*, The latency histogram was constructed out of synaptic currents occurred after 1094 stimuli during tetani.

cAMP, at 0.86 mM. When the responses from seven control larvae (line 34) were averaged, dibutyl cAMP transiently increased the frequency of mESCs between 8 and 22 min of application, although the time course and amplitude of the response was somewhat variable for cell to cell. The averaged frequency at the peak 10 min after application of the drug was $17.1 \pm 30.2/\text{min}$ ($n = 7$, three consecutive points, 1 min per point, were averaged) (Fig. 8*Ba*). In contrast, in *n-syb*^{ΔF33B} embryos no response was observed in all six cells examined (Fig. 8*Bb*). Similarly, in *n-syb*^{F33-R} no response was observed in all six cells examined (Fig. 8*Bc*). Thus, the response to dibutyl cAMP was completely absent in *n-syb* mutant embryos. The response to stimulation of the cAMP pathway appears to depend absolutely to the presence of *n-syb*.

DISCUSSION

v-SNARE proteins such as synaptobrevin are thought to be essential in membrane trafficking. In this study, we have observed that vesicles retain the ability to fuse both spontaneously and in response to elevated levels of cytosolic Ca²⁺. These fusions could occur in a pathway that is independent of a v-SNARE protein or, alternatively, the fusions may involve another v-SNARE: the homologous but largely non-*n-syb* gene, an additional homolog

that has not yet been discovered, or perhaps vesicular syntaxin acting as a v-SNARE. The purpose of this study was to probe this *n-syb*-independent pathway to learn its capacities and limitations. Although this pathway could not respond to the Ca²⁺ transient elicited by an individual action potential, we found that it was still capable of regulation by Ca²⁺. In contrast, the *n-syb*-independent pathway was unresponsive to cAMP.

n-syb null mutants retain Ca²⁺-dependent release

In *n-syb*^{ΔF33B} and *n-syb*^{F33-R} embryos, we found that nerve stimulation at 0.3 Hz did not evoke synaptic currents under any circumstances we tried. To determine whether the *n-syb*-independent pathway could respond to changes in cytosolic Ca²⁺ other than action potential-triggered changes, we studied the effects of depolarizing the terminal with high K⁺ salines. We demonstrated that the mESC frequency was increased in an external Ca²⁺-dependent manner, even in *n-syb*^{ΔF33B}, which is unequivocally a null allele (Deitcher et al., 1998). Thus Ca²⁺ ions entered the terminal as the membrane was depolarized, and this influx was blocked by the Ca²⁺ channel blockers Cd²⁺ and PLTX-II. These observations are in accord with a previous report in synaptosomes, in which an increase in internal Ca²⁺ was

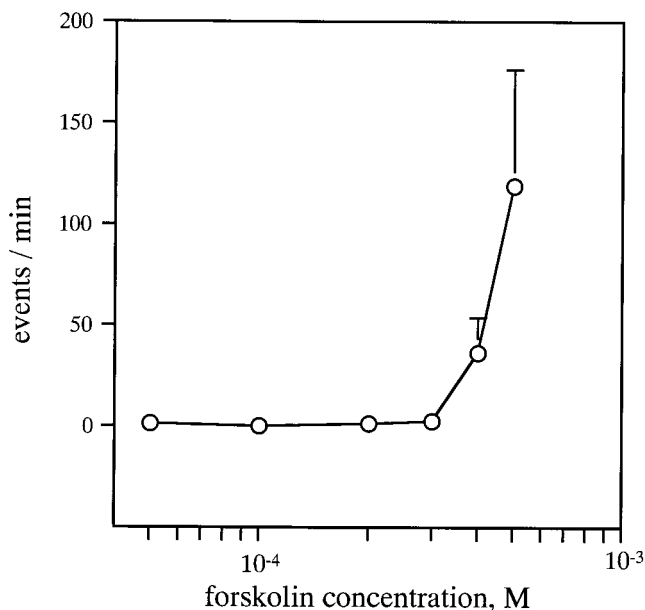


Figure 7. Effects of forskolin on the mESC frequency in line 34 larvae. The concentration-dependent increase of mESC frequency is shown in the ordinate as the difference of mESC events per minute between the average of frequency during a 5 min interval shortly after application of forskolin and that of a 5 min interval between 25 and 30 min after drug application. Data from three cells for 50 μM , from three cells for 100 μM , from six cells for 200 μM , from three cells for 300 μM , from six cells for 400 μM , and from four cells for 500 μM were averaged. Error bars indicate SEM.

induced by K^+ stimulation despite the presence of tetanus toxin (McMahon et al., 1992).

Although the rate of release in the *n-syb* mutant did not match that of wild-type, this difference cannot be attributed to an impairment of Ca^{2+} channel function because the same diminution of release was observed when the Ca^{2+} channels were bypassed with the ionophore A23187 or with black widow spider venom. We therefore ascribe the downward shift in the curve relating Ca^{2+} to mESC frequency to a decrease in the efficiency of transmitter release.

The decrease in mESC frequency in the *n-syb* mutants did not correlate with the complete absence of a response to an individual action potential, even when the Ca^{2+} influx in response to the action potential had been augmented by high Ca^{2+} concentrations or 4-AP (Deitcher et al., 1998). This discrepancy raised the possibility that there was a change in the kinetics of the Ca^{2+} channel that prevented its gating by an action potential. This possibility also appears to be excluded by studies of tetanic stimulation: the long-lasting increase in mESC frequency that was observed during and after the tetani confirmed that action potentials could raise cytosolic Ca^{2+} . The latency of these events was not compatible with a decrease in the probability of channel opening; such a decrease would have produced normal latency responses, as was seen in wild-type synapses in low Ca^{2+} .

***n-syb* null mutants do not respond to an elevation of cAMP**

In the absence of external Ca^{2+} , forskolin evoked marked transmitter release in line 34 larvae. Dibutyryl cAMP had a similar, although less robust, effect. In the *n-syb* null allele, these responses were entirely absent. cAMP probably does not act in these synapses by releasing Ca^{2+} from intracellular stores: such

elevations would, like those evoked by high K^+ or ionophores, be expected to act in the absence of *n-syb*. Instead, cAMP would appear directly to facilitate the fusion of synaptic vesicles. This action could be accomplished by increasing the number of fusion-competent docked vesicles, by altering the fusion apparatus to increase the probability of release, or by altering a Ca^{2+} sensor so that it was capable of responding to resting Ca^{2+} levels. These observations are consistent with studies in rat cerebellar slices in which forskolin increases the mESC frequency without raising internal Ca^{2+} (Chen and Regehr, 1997).

Unlike Ca^{2+} -dependent stimulation of release, which was only partially inhibited by loss of *n-syb*, cAMP-dependent stimulation was completely removed. The *n-syb* product is therefore essential to the processes of synaptic facilitation and post-tetanic potentiation that involve cAMP (Zhang et al., 1999a,b; Weisskopf et al., 1994). Although very little is known about the intracellular action of cAMP at synapses, the *n-syb* phenotype suggests that a phosphorylation target of protein kinase A could be either *n-syb* itself or a protein upstream of *n-syb* in the exocytotic apparatus.

Plausible models for the *n-syb* phenotype

What then is the critical difference between the *n-syb*-mediated and the *n-syb*-independent pathways for Ca^{2+} -dependent release? What allows only the former to respond to an individual action potential? One indication may come from the time course of the response to tetanic stimulation. The latency histogram suggested a slow step in the coupling of Ca^{2+} entry to fusion. Two attractive models present themselves at present.

The first model invokes a difference in the spatial relationship of the active Ca^{2+} channels to the fusion-competent vesicles. Specifically, in the absence of *n-syb*, the vesicles would no longer be anchored in the immediate vicinity of the channels. Thus, in the null mutants, the brief and focal rise in Ca^{2+} concentration would dissipate because of diffusion and buffering before reaching the Ca^{2+} sensor. A similar spatial separation has been invoked to explain the Ca^{2+} sensitivity and time course of release in chromaffin cells (Chow et al., 1996). Moreover, such an explanation would be consonant with known biochemical properties of mammalian synaptic proteins. Syntaxin binds to synaptic isoforms of Ca^{2+} channels, and vesicle-associated membrane protein–synaptobrevin binds to syntaxin (Söllner et al., 1993a,b). Thus, the absence of *n-syb* could prevent the association of the vesicle with the channel while leaving the vesicle free to sense Ca^{2+} and fuse with the membrane when sustained Ca^{2+} influx allowed the ions to reach the sensor. A variant of this hypothesis would invoke two classes of Ca^{2+} channel in the terminal. The class that normally dominates release can be inhibited by syntaxin (Bezprozvanny et al., 1995). *n-syb* protein, by binding to syntaxin, could dissociate it from the channel and thereby allow it to trigger fusion when opened by an action potential. In the absence of *n-syb*, fusion would have to rely on more distant channels that are not inhibited by syntaxin.

The second model invokes two distinct Ca^{2+} sensors. In this model, the *n-syb*-mediated pathway is coupled to a low-affinity Ca^{2+} sensor and leads to rapid fusion. The *n-syb*-independent pathway is coupled to a high-affinity Ca^{2+} sensor, but the rate at which Ca^{2+} binding leads to vesicle fusion is slower than in the *n-syb*-dependent pathway. Evidence from several systems has already pointed to the existence of two classes of Ca^{2+} sensor. After tetanic stimulation, evoked release is enhanced because of residual Ca^{2+} binding to high-affinity sites (micromolar range) that, perhaps because of slow kinetics, are rarely occupied by

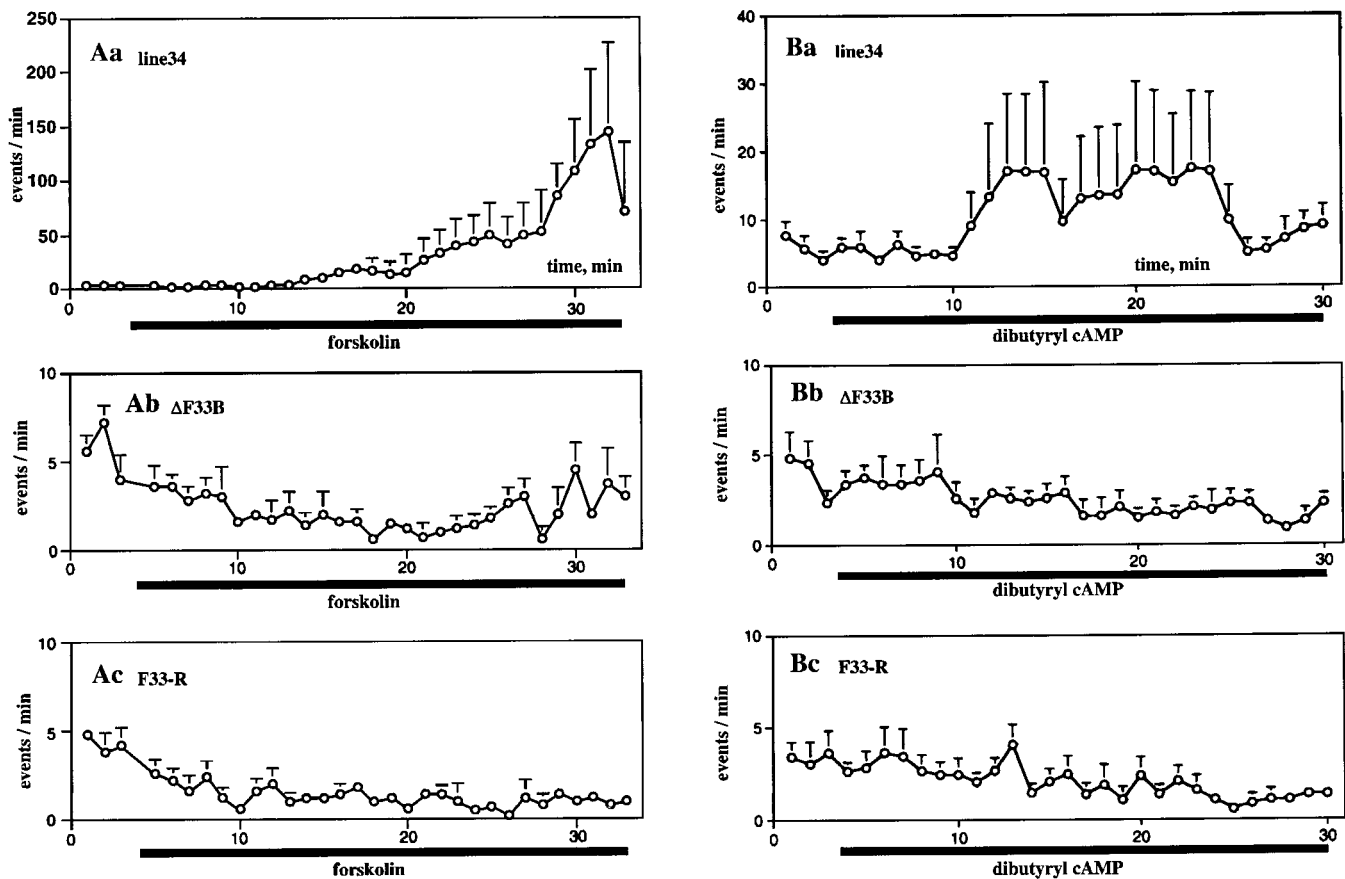


Figure 8. Effects of forskolin (*A*) and dibutyryl cAMP (*B*) on the mESC frequency in the absence of external Ca^{2+} . Forskolin ($500 \mu\text{M}$) or dibutyryl cAMP (0.86 mM) was applied during a horizontal bar below the abscissa. *Aa* and *Ba* are from line 34 (data from 4 cells for *Aa* and 7 cells for *Ba* were averaged). Error bars indicate SEM. *Ab* and *Bb* are from *n-syb* $^{\Delta\text{F33B}}$ (data from 5 cells for *Ab* and from 6 cells for *Bb* were averaged). *Ac* and *Bc* are from *n-syb* $^{\text{F33-R}}$ (data from 5 cells for *Ac* and from 4 cells for *Bc* were averaged).

Ca^{2+} entering during a single action potential. These high-affinity sites are distinct from the low-affinity sites that are occupied only when a high concentration of cytosolic Ca^{2+} is achieved after the arrival of an action potential (Yamada and Zucker, 1992; Kamiya and Zucker, 1994; van der Kloot, 1994).

Occupation of high-affinity sites may be sufficient to cause spontaneous fusion of synaptic vesicles. Although the frequency of mESCs has not yet been correlated directly to presynaptic Ca^{2+} , conditions that are likely to raise Ca^{2+} to the 1–3 μM range appear to promote spontaneous fusions (Miledi and Thies, 1971; Erulkar and Rahamimoff, 1978; Kamiya and Zucker, 1994). Therefore, the low-affinity sites need not be occupied for spontaneous release of transmitter to occur, albeit at a relatively slow rate. To accomplish the rapid release of vesicles evoked by an action potential, the low-affinity sites must be occupied as well, which occurs only in the microenvironment of the open Ca^{2+} channel.

We would add to this description that the high- and low-affinity sites appear to trigger distinct fusion mechanisms: only the slow, high-affinity site can operate in the absence of *n-syb*. The Ca^{2+} sensor for the *n-syb*-independent pathway would largely go unoccupied and would not trigger release after an individual action potential. This pathway would be triggered by sustained Ca^{2+} increases caused by tetanic stimulation, high K^+ , or Ca^{2+} ionophores, but does not appear to be modulated by cAMP.

Exocytosis in secretory cells has been divided into constitutive

and regulated pathways (Kelly, 1988). The genetic ablation of the evoked ESC and persistence of mESCs might have reflected the ability of the constitutive pathway to mediate a portion of the spontaneous release. The present experiments, however, indicate that this spontaneous release arises from a Ca^{2+} -regulated pathway that is genetically distinguishable from the evoked pathway.

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