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Mutational Analysis of Drosophila *synaptotagmin* Demonstrates Its Essential Role in Ca^{2+}-Activated Neurotransmitter Release

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Summary

Synaptotagmin (syt), a synaptic vesicle–specific protein known to bind Ca^{2+} in the presence of phospholipids, has been proposed to mediate Ca^{2+}-dependent neurotransmitter release. We have addressed the role of syt in neurotransmitter release in vivo by generating mutations in *synaptotagmin* (*syt*) in the fruitfly and assaying the subsequent effects on neurotransmission. Most embryos that lack syt fail to hatch and exhibit very reduced, uncoordinated muscle contractions. Larvae with partial lack-of-function mutations show almost no evoked excitatory junctional potentials (EJPs) in 0.4 mM Ca^{2+} and a 15-fold reduction in EJP amplitude in 1.0 mM Ca^{2+} when compared with heterozygous controls. In contrast, we observe an increase in the frequency of spontaneous miniature EJPs in the mutants. These results provide in vivo evidence that syt plays a key role in Ca^{2+} activation of neurotransmitter release and indicate the existence of separate pathways for evoked and spontaneous neurotransmitter release.

Introduction

Communication between neurons and their targets at a synapse is initiated by release of neurotransmitter from presynaptic terminals (Del Castillo and Katz, 1956; Miledi, 1973). This release is thought to be based exclusively on the fusion of synaptic vesicles with the presynaptic cell membrane (Ceccarelli and Hurlbut, 1980; Heuser et al., 1979). Neurotransmission is initiated by depolarization of the presynaptic terminal that allows influx of extracellular Ca^{2+} via voltage-gated Ca^{2+} channels. The influx of Ca^{2+} is absolutely required for evoked neurotransmitter release (Mulkey and Zucker, 1991). In both squid giant axons and mammalian neurons, the lag between Ca^{2+} influx and postsynaptic effect is approximately 200 μs (Llinas et al., 1981; Cope and Mendell, 1982). The requirement for Ca^{2+} and the speed of the process have led to the conclusion that Ca^{2+} must directly and rapidly activate the machinery involved with fusion of synaptic vesicles with the presynaptic membrane.

Structural data provide additional clues to the mechanisms of neurotransmitter release. Electron microscopy shows that vesicle fusion is restricted to specialized release sites on the presynaptic terminal. At the neuromuscular junction, these sites are arranged along an electron-dense bar that directly aligns sites of release with receptors on the postsynaptic cell (Kuffler et al., 1984). Freeze-fracture electron microscopy shows rows of intermembraneous particles arranged along these release sites (Heuser et al., 1979). These particles have been suggested to represent presynaptic voltage-gated Ca^{2+} channels (Cohen et al., 1991). The possible conjunction of release sites and Ca^{2+} channels stimulated mathematical modeling of Ca^{2+} concentrations at release sites when presynaptic terminals are depolarized. These studies suggest that very high Ca^{2+} concentrations, on the order of 10–500 μM, may be responsible for synaptic vesicle fusion (Augustine et al., 1987; Simon and Llinas, 1985). Recent work using modified forms of the Ca^{2+} indicator aequorin in the squid giant synapse shows that such microdomains of high Ca^{2+} concentration exist (Llinas et al., 1992).

The requirement for high Ca^{2+} concentrations for neurotransmitter release and the rapid membrane fusion of synaptic vesicles has guided the search for synaptic vesicle and presynaptic proteins that may mediate this release (for review see Südhof and Jahn, 1991). The transmembrane synaptic vesicle protein synaptotagmin (syt) has been a particular focus because it contains domains involved with Ca^{2+}-dependent membrane interaction (Perin et al., 1990, 1991a, 1991b) and binds Ca^{2+} in a cooperative manner in the presence of negatively charged phospholipids at Ca^{2+} concentrations of 10–100 μM (Brose et al., 1992). Syt contains a short intravesicular sequence, a single transmembrane domain, and a large cytoplasmic region mostly consisting of two repeats that have sequence homology to the C2 domain of protein kinase C (Perin et al., 1990; Nishizuka, 1989). These properties have led to speculation that syt may be the Ca^{2+} receptor for exocytosis.

Biochemical experiments have suggested specific interactions of syt with presynaptic proteins thought to be important for docking or modulation of neurotransmitter release. Of particular interest is the interaction with the latrotoxin receptor, a member of the neurexin family (Ushkaryov et al., 1992; Petrenko et al., 1991). This interaction may represent a mechanism for vesicle docking or modulation of neurotransmitter release since latrotoxin causes massive exocytosis even in the absence of external Ca^{2+} (Matteoli et al., 1988). Syt has also been reported to interact with the presynaptic protein syntaxin (Bennett et al., 1992) and voltage-gated Ca^{2+} channels (Leveque et al., 1992). The precise role for any of these interactions in vivo has yet to be demonstrated.

One approach to addressing the specific role of syt in docking and fusion is to mutate or remove the protein and assess the consequences on neurotransmission. Two recent reports have presented evidence that mutations in *synaptotagmin* (*syt*) in Drosophila melanogaster (DiAn-

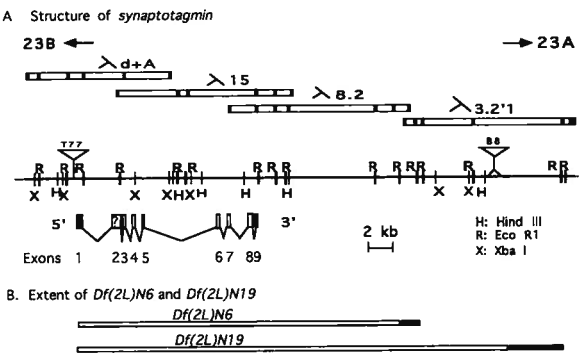

Figure 1. Structure of the *syt* Locus

(A) λ dash phages covering the *syt* locus are indicated above the restriction map. These phages map to cytological bands 23A6–23B1. The closed bars in the phage inserts correspond to EcoRI sites. Identified exons of the *syt* pD65-1 cDNA are shown below the restriction map. Noncoding regions are indicated by closed boxes; open boxes indicate coding sequences. An additional 5.4 kb of 3′ untranslated coding sequences was not mapped. The mapping position of the P[*lacZ,w*]B8 insertion that was mobilized to isolate the P[*lacZ,w*]T77 insertion in *syt* is shown. *syt* is transcribed from the centromere toward the distal end of the left arm of the second chromosome.

(B) The extent of two imprecise P[*lacZ,w*]T77 excisions (*Df(2L)N6* and *Df(2L)N19*) are indicated. *Df(2L)N6* and *Df(2L)N19* do not affect the region upstream of *T77*, but remove parts of the 3′ portion of the insertion as well as the open reading frame of the *syt* locus. The *B8* insertion is not affected in *Df(2L)N6* and is excised in *Df(2L)N19*. The closed bars correspond to EcoRI genomic fragments in which the deficiencies break.

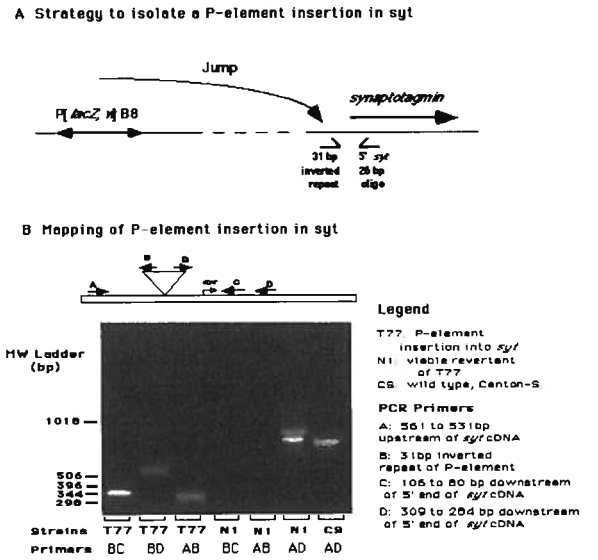

Figure 2. P Element Mutagenesis

(A) Strategy used to isolate insertions into *syt*. The *B8* insertion was mobilized by introduction of Δ2-3 transposase.

(B) PCR analysis used to map the *T77* insertion. The P element insertion into *syt* was mapped with PCR in *T77/Gla* flies using primers from the P element (primer B) and the 5′ region of *syt* (primers A, C, and D). The insertion maps approximately 250 bp upstream of the longest cDNAs, probably in regulatory sequences of *syt* (90 bp upstream of long stretches of GAGA). PCR analysis of a viable revertant, *N1*, is also shown. The *N1* revertant lacks the *T77* insertion and shows no evidence of improper excision as revealed by PCR with primers A and D, which flank the insertion.

tonio et al., 1993) and Caenorhabditis elegans (Nonet et al., 1993) alter synaptic function but still allow some release of neurotransmitter. To address the role of syt more precisely, we have isolated another set of mutations in *syt* in Drosophila. Here, we describe the consequences of loss of syt in embryos and first instar larvae. In addition, we present electrophysiological recordings at the neuromuscular junction of third instar larvae that carry partial lack-of-function alleles. These studies demonstrate that syt plays a key role in Ca^{2+}-dependent evoked neurotransmitter release. In addition, our data indicate the presence of independent release mechanisms that underlie evoked and spontaneous release.

Results

Genomic Structure and Targeted Insertion into the *syt* Locus

We have recently presented evidence that a single *syt* gene located at cytological band 23B encodes syt in Drosophila. Syt is expressed by all neurons and is localized specifically to synapses (Littleton et al., 1993). To initiate a mutational analysis of *syt*, genomic sequences at and around the *syt* locus were isolated, and the structure of the locus was determined. The exon–intron boundaries were mapped using *syt* cDNA pD65-1 (Perin et al., 1991a), which is approximately 2.0 kb long and contains the entire open reading frame (open boxes in Figure 1A). However, Northern analyses show that some *syt* transcripts are as large as 7.4 kb (Perin et al., 1991a; Littleton et al., 1993). Molecular analyses indicate that most of the additional 5.4 kb is part of the 3′ untranslated domain (data not shown). Hence, the locus must extend more than 5 kb 3′ of the mapping positions shown in Figure 1A.

Knowledge of the genomic structure around *syt* allowed us to map a P element, P[*lac,w*]B8 (a gift from Y. N. Jan and G. Feger), about 40 kb downstream of the 5′ end of *syt*. Homozygous P[*lac,w*]B8 flies are viable and show no obvious morphological or behavioral phenotypes. In addition, the β-galactosidase expression pattern of this enhancer detector strain is different from the previously described *syt* expression pattern (Littleton et al., 1993). These data indicate that P[*lacZ,w*]B8 is not inserted in *syt* but that it is at most 10 kb downstream of the 3′ end of the transcript.

We mobilized the P[*lacZ,w*]B8 P element into *syt* by combining the local hopping strategy described by Tower et al. (1993) and the polymerase chain reaction (PCR) methodology described by Kaiser and Goodwin (1990) (see Figure 2A). We screened 3200 independent events and found a new insertion, P[*lac,w*]T77, which maps 250 bp 5′ of the longest *syt* cDNA (Figure 2B). This insertion, abbreviated *T77*, causes homozygous lethality. Precise or near-precise excision of *T77* reverts the lethality (see excision *N1* in Figure 2B). Homozygous *T77/T77* embryos have very reduced levels of *syt* transcripts and syt protein (see below). These data indicate that the *T77* insertion

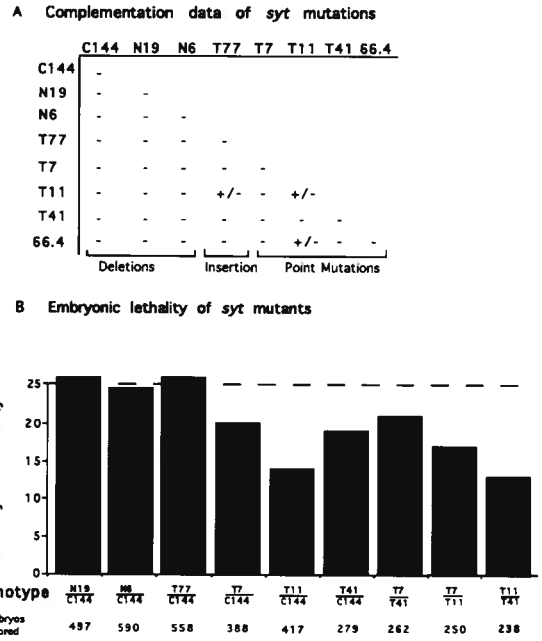

Figure 3. Genetic Analysis and Embryonic Lethality of *syt* Mutants

(A) Complementation table of *syt* alleles (the minus sign indicating lethal, with plus/minus indicating less than 10% expected offspring). All identified *syt* mutations form a single complementation group. Three combinations of partial lack-of-function alleles produce viable offspring that exhibit severe behavioral defects (see text).

(B) Embryonic lethality of *syt* alleles. Combinations of *syt* null alleles, *Df(2L)N19* and *Df(2L)N6*, and *Df(2L)C144* exhibit 25% embryonic lethality. Combinations of some EMS-induced alleles, e.g., *T7* and *T41* with *Df(2L)C144* and transheterozygous *T7/T41*, exhibit 20% embryonic lethality. Lethality was scored 48 hr after egg laying.

causes a lethal mutation in *syt* that can be reverted by excision of the P element.

Lack and Partial Lack-of-Function Alleles of *syt*

To generate *syt* null alleles, imprecise excisions of *T77* were generated using the Δ2-3 transposase strategy. Approximately 300 balanced lines were established on the basis of eye color reversion. Using sets of PCR primers at the 5′ (primers A and B) and 3′ (primers B and D) (Figure 2B) end of the *T77* P element, along with Southern analysis, two imprecise excisions were selected and characterized in more detail. As shown in Figure 1B, *Df(2L)N6* removes the entire open reading frame of the *syt* locus, whereas *Df(2L)N19* removes all the genomic DNA between the *T77* and the *B8* insertions. Both mutations cause embryonic lethality, and homozygous *N6* and *N19* embryos lack *syt* transcripts and syt protein (see below).

To generate more subtle mutations in *syt*, a chemical mutagenesis using ethyl methanesulfonate (EMS) was initiated. Approximately 60 EMS-induced alleles that fail to complement *Df(2L)DTD2* were recovered from about 4000 chromosomes screened. This deficiency uncovers cytological bands 22D4-5;23B1-2 (Spencer et al., 1982) and also uncovers *syt* (J. T. L. et al., unpublished data). As shown in Figure 3A, three of these EMS-induced mutations (*T7*, *T11*, and *T41*) and another independently isolated *syt* allele named *66.4* (a gift from W. Gelbart and J. Sekelsky) failed to complement each other as well as the lethality caused by the *T77*, *N6*, and *N19* alleles. The four EMS-induced alleles were analyzed by genomic Southern analysis and showed no obvious rearrangements in the cloned genomic fragments shown in Figure 1A, indicating that they are point mutations or subtle rearrangements in *syt*. In addition, the complementation data clearly show that three alleles (*T11*, *T77*, and *66.4*) are partial lack-of-function alleles since a few adult survivors were observed in transheterozygous combinations. It is likely that the *T11* allele is the least severe allele of the three, as homozygous *T11* flies are viable.

Molecular Defects Caused by *syt* Mutations

To characterize the molecular defects caused by the various mutations, mutant strains were analyzed by whole-mount in situ hybridization using digoxigenin-labeled DNA or RNA *syt* probes. As summarized in Table 1 and shown in Figure 4, homozygous *Df(2L)N6* and *Df(2L)N19* embryos lack *syt* message, whereas heterozygous and wild-type embryos express *syt* abundantly in cells of the central nervous system and in all neurons of the peripheral nervous system (Littleton et al., 1993). Homozygous *T77* embryos exhibit very reduced but detectable levels of transcripts in the nervous system (data not shown). The EMS-induced alleles do not cause obvious differences in the levels of *syt* transcripts. It should be noted that only substantial differences in transcript levels will be detected with this technique.

Table 1. Properties of *syt* Mutations

Allele	Type of Mutagen	Genomic Structure[a]	Transcript Levels[b]	Protein Levels[c]	Type of Allele
T77	Insertion	Altered	Very reduced	Very reduced	Strong hypomorph
N6	Imprecise excision	Altered	Not detected	Not detected	Null
N19	Imprecise excision	Altered	Not detected	Not detected	Null
T7	EMS	Wild type	Present	Not detected	Null/severe hypomorph
T11	EMS	Wild type	ND[d]	Present	Weak hypomorph
T41	EMS	Wild type	Present	Variable	Null/severe hypomorph
66.4	EMS	Wild type	ND[d]	Present	Hypomorph

[a] Determined by Southern analysis.
[b] Determined by whole-mount in situ hybridization.
[c] Determined by immunocytochemical staining with polyclonal antibody DSYT2.
[d] Not determined.

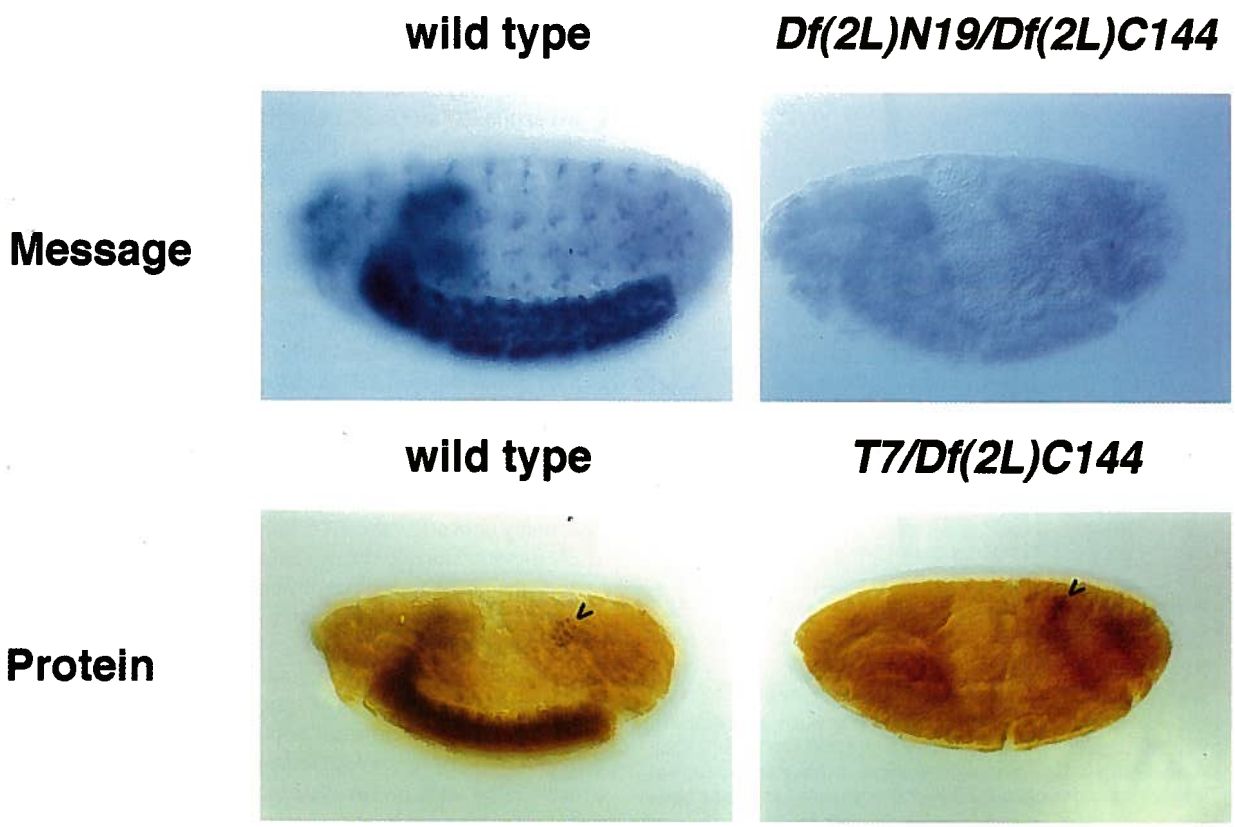

Figure 4. Molecular Defects in *syt* Mutants

All embryos are shown dorsal up, anterior to the left.

(Top panels) Late stage 16 whole-mount embryos hybridized in situ with a digoxigenin-labeled *syt* cDNA. (Left) Wild-type embryo. (Right) *Df(2L)N19/Df(2L)C144* embryo. Mutant embryos completely lack *syt* message in both the central and peripheral nervous systems.

(Bottom panels) Stage 17 whole-mount embryos immunocytochemically stained with polyclonal antibody DSYT2 prepared against the cytoplasmic domain of Drosophila syt (Littleton et al., 1993). (Left) Wild-type embryo. (Right) *T7/Df(2L)C144* embryo. Note the absence of protein in the central nervous system of the *T7/Df(2L)C144* embryo. Also note the presence of label in the gonads of both embryos, indicated by the arrow. We have previously shown that this cross-reacting gonadal epitope is recognized by the DSYT2 antibody (Littleton et al., 1993). This gonadal staining serves as a positive control, demonstrating that both embryos were exposed to primary and secondary antibodies.

To determine whether *syt* mutants produced protein and whether syt is properly localized to synapses, we stained embryos immunocytochemically with polyclonal antibody DSYT2, which was raised against the cytoplasmic domain of Drosophila syt (Littleton et al., 1993). As shown in Figure 4 and Table 1, *T7/Df(2L)C144* and *T7/T7* embryos lack detectable levels of syt, whereas *T77/T77* embryos exhibit very reduced levels of protein. In addition, *T41/T41* embryos have reduced and variable levels of syt. The remainder of the mutants either do not contain syt protein at detectable levels (*N19* and *N6*) or cannot be distinguished from wild-type embryos (*T11* and *66.4*). These data corroborate the complementation data.

Phenotypic Analysis of *syt* Mutants

Wild-type embryos exhibit coordinated sets of muscle contractions prior to hatching from the egg case during late stage 16 and stage 17 (Broadie and Bate, 1993). These contractions are required for hatching and motility of the first instar larvae. As shown in Figure 3B, a quarter of all embryos derived from a cross of *Df(2L)N6/+* (the plus sign indicates Canton-S) and *Df(2L)C144/+* flies die. Similar results were also obtained for two other transheterozygous mutant combinations (see Figure 3B). In addition, approximately 80% of the *T7/Df(2L)C144*, *T41/Df(2L)C144*, and *T7/T41* embryos die. The observation that *T7/T41* embryos are as severely affected as *T7/Df(2L)C144* and *T41/Df(2L)C144* embryos suggests that *T7* and *T41* are null alleles. Failure to detect syt in *T7/T7* embryos also suggests that *T7* may be a null allele. Because the embryonic lethality of *Df(2L)N6/Df(2L)C144* or *Df(2L)N19/Df(2L)C144* embryos is more extreme than the phenotype of *T7/T41* embryos (100% versus 80%), *Df(2L)N6* and *Df(2L)N19* may affect another gene. Germline rescue of *Df(2L)N6* and *Df(2L)N19* will be required to determine whether they affect another gene. These data also suggest that *Df(2L)C144* does not enhance the phenotype of *syt* mutants, even though at least five genes are uncovered by this deficiency (J. T. L. et al., unpublished data). In addition, the embryonic lethality of embryonic combinations with allele *T11* indicates that it is a recessive partial loss-of-function allele. Since *T77/T11* and *66.4/T11* flies are viable but other allelic combinations of *T77* and *66.4* are not viable, we propose the following allelic series, from most severe to least se-

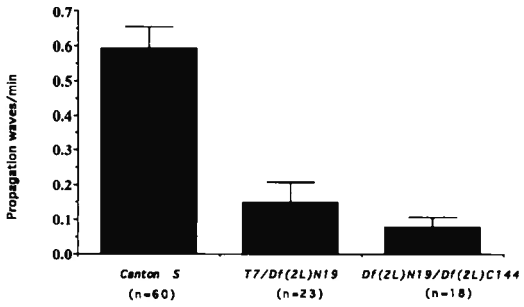

Figure 5. Reduced Contractions in *syt* Mutant Embryos

Embryos lacking syt protein (*Df(2L)N19/Df(2L)C144* and *T7/Df(2L)N19*) show a dramatic reduction in coordinated peristaltic muscle contractions measured 21–22 hr after egg laying at 23°C ± 1°C. Error bars indicate SEM. The number of embryos scored in 5 min intervals for each genotype is indicated. Peristaltic contractions were significantly reduced when compared with wild-type embryos (0.59 ± 0.06 SEM) in both *T7/N19* (0.15 ± 0.06 SEM) and *N19/C144* (0.08 ± 0.03 SEM) embryos (unpaired Student's t test, $p < 0.001$). There is no statistically significant difference in contractions displayed in *T7/N19* and *N19/C144* embryos.

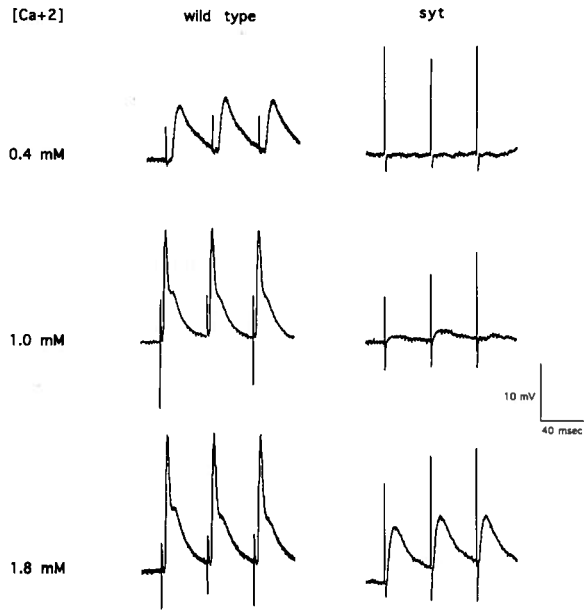

Figure 6. Intracellular Recordings of Muscle EJPs Evoked by Three Nerve Stimulations at a Frequency of 20 Hz, at the Indicated External Ca^{2+} Concentration

Representative traces are shown. The traces on the left are from larvae of genotype *T11/Gla Bc*; such larvae are phenotypically *syt*$^+$. The traces on the right are from larvae of genotype *T11/T77*; such larvae are phenotypically *syt*$^-$. The scale for the voltage trace is 10 mV; the scale for the sweep speed is 40 ms.

vere allele: *N19 = N6 ≥ T7 = T41 > T77 = 66.4 > T11*. These results are summarized in Table 1 and indicate that all alleles are loss-of-function mutations.

Complete absence of *syt* in *Df(2L)N6/Df(2L)C144* or *Df(2L)N19/Df(2L)C144* causes failure to hatch of approximately 100% of the embryos (25% of the embryos of the cross *Df(2L)N6/+ × +/Df(2L)C144*) 48 hr after egg laying (normal embryos hatch after 22–24 hr). These embryos show a severe reduction in coordinated muscle contractions when compared with wild-type embryos. They also exhibit uncoordinated twitching of muscle fibers within individual segments. To quantitate these muscle contractions, we counted the propagation waves per minute in wild-type and mutant embryos at 21–22 hr of development (Figure 5). A 5-fold reduction in the number of propagation waves was observed in transheterozygous mutant combinations that lack syt protein. We cannot rule out the possibility that *Df(2L)N6* and *Df(2L)N19* may remove another essential gene next to *syt* that contributes somewhat to the defects that we observe. However, mutant embryos that carry EMS-induced point mutations classified as null alleles or severe lack-of-function mutations (e.g., *T7/Df(2L)C144;T7/T41*) fail to hatch in about 80% of the cases. The remaining 20% of embryos are able to elicit brief more or less coordinated contractions and to free themselves from the egg case before dying as first instar larvae. The 80% of the embryos that do not hatch can be freed from the egg case. These embryos are immobile but again show some contractions. We conclude that embryos that lack syt function are embryonic lethal but are able to elicit some abnormal and reduced muscle contractions, suggesting that there may be one or more independent pathways that can result in a greatly reduced release of transmitter in the absence of syt.

As mentioned previously, some homozygous and transheterozygous mutant flies (*T11/T11*, *T77/T11*, and *66.4/T11*) eclose. These flies carry combinations of partial lack-of-function alleles. Surviving third instar larvae that develop into these flies are sluggish and display abnormal movements. They are unable to crawl up the sides of their containers, even though they are essentially normal in size and otherwise look healthy. Occasionally, they undergo slow and deliberate feeding behavior and have a characteristic maintained protrusion of the mouth hooks. These larvae do respond to physical stimulation, although in a severely delayed and reduced manner. They develop into flies that typically eclose a few days later than their littermates. These flies fail to unfold their wings after eclosion and show very severe behavioral defects as they are unable to walk more than a few steps without falling over and are unable to mate, jump, fly, or feed. These flies also show severely uncoordinated leg movements and uncoordinated thoracic and abdominal body wall contractions. Most flies die within 48 hr after eclosion.

Partial Loss-of-Function *syt* Alleles Show Severe Reductions in Ca^{2+}-Induced Neurotransmitter Release

The ability to obtain a few viable third instar larvae derived from certain allelic combinations of *syt* (homozygous *T11* or *T11/T77*) enabled us to test the effects of a partial lack of syt on synaptic transmission using the larval neuromuscular preparation developed by Jan and Jan (1976). This preparation permits the recordings of muscle depolarizations (termed the excitatory junctional potential [EJP]) elicited by nerve stimulation and consequent transmitter re-

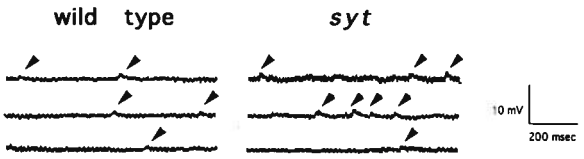

Figure 7. Intracellular Recordings of Spontaneous Muscle Depolarizations

MEJPs are indicated by arrows. The external Ca^{2+} concentration was 1.0 mM. The traces on the left are from larvae of genotype *T11/Gla Bc*; such larvae are phenotypically *syt*$^+$. The traces on the right are from larvae of genotype *T11/T77*; such larvae are phenotypically *syt*$^-$. The scale for the voltage trace is 10 mV; the scale for the sweep speed is 200 ms.

lease. In addition, muscle depolarizations resulting from the spontaneous release of individual synaptic vesicles (termed the miniature excitatory junctional potentials [MEJPs]) can also be monitored with this preparation. As described previously, transmitter release at the wild-type larval neuromuscular junction is strongly Ca^{2+} dependent (Jan and Jan, 1976). At an external Ca^{2+} concentration of 0.1 mM, transmitter release cannot generally be evoked and no EJP occurs (data not shown). However, increases in external Ca^{2+} concentration lead to increases in transmitter release and in EJP amplitude (see Figure 6; see Table 2) so that at an external Ca^{2+} concentration of 1.8 mM, an EJP of about 26 mV is obtained.

We find that *syt* mutants display a very striking reduction in evoked transmitter release compared with wild type. Figure 6 shows the properties of the *T11/T77* transheterozygote; larvae homozygous for allele *T11* display similar defects. It should be noted that all mutant larvae tested exhibit very similar responses (see Table 2). For example, at an external Ca^{2+} concentration of 0.4 mM, transmitter release generally cannot be elicited at all. Only 2 of 5 larvae tested at this concentration responded and only then with release of a single quanta compared with an average of at least 19 quanta (mean EJP/MEJP amplitude) released in *T11/Gla Bc* controls. However, repetitive stimulation at high frequency (10–20 Hz) sometimes allows some additional transmitter release to occur (data not shown). This phenomenon, termed facilitation, has been attributed to a build up of Ca^{2+} in the nerve terminal (Katz and Miledi, 1967). Facilitation is also observed at the wild-type larval neuromuscular junction, but only at an external Ca^{2+} concentration of 0.2 mM or less (Jan and Jan, 1978). These results strongly suggest that at low external Ca^{2+} concentration in which the wild-type synapse is functional, Ca^{2+}-evoked release is absent in the *T11/T11* and *T11/T77* mutant larvae. Since the EMS-induced *T11* allele and the P element insertion *T77* were derived from completely different genetic backgrounds, it is highly unlikely that the two chromosomes would contain an additional shared mutation that would contribute to this effect.

Evoked release in *syt* mutants is still Ca^{2+} dependent; when the external Ca^{2+} concentration is raised to 1.0 mM, a very small amount of transmitter release can occur that is approximately 15-fold lower in amplitude than wild type (Figure 6). Transmitter release could only be partially restored at a high external Ca^{2+} concentration of 1.8 mM. However, the response is delayed and reduced 4-fold compared with wild-type reponses and shows some similarities to responses seen in wild-type larvae at a 0.4 mM Ca^{2+} concentration. We expect that the *T11* allele is still capable of some function in high Ca^{2+}, allowing for the release that we observe (see Discussion).

In contrast with the striking effect of *syt* mutations on evoked transmitter release, *syt* mutations conferred no decrease in either the rate or amount of spontaneous transmitter release as assayed by the rate and amplitude of MEJPs. In fact, MEJP frequency was increased about 2-fold in *syt* mutants (Figure 7; Table 2). Heterozygous *T11/Gla Bc* control larvae had a mean value of 1.45 ± 0.33 MEJPs per second (combined data for all three Ca^{2+} concentrations, which were not statistically significantly different) compared with 3.02 ± 0.41 MEJPs per second in *syt* mutants (unpaired Student's t test, $p < 0.01$). MEJP amplitude was not affected in *syt* mutants (0.71 ± 0.1 mV in control compared with 0.68 ± 0.1 mV in mutants), and both mutant and wild-type muscles have resting potentials of about −50 mV, demonstrating the integrity of the postsynaptic membrane in mutant larvae. These results show that the *syt* mutant muscle responds normally to released neurotransmitter, indicating that the reduction in EJP is of presynaptic origin. Prolonged recordings (>15 min) of spontaneous MEJPs in mutant larvae show no time-dependent exhaustion of this release pathway, suggesting that vesicle recycling is not affected. Heterozygous *Df(2L)N6/Gla Bc* and *T11/Gla Bc* showed no obvious defects in responses compared with Canton-S control larvae (Jan and Jan, 1976), ruling out possible dominant effects caused by a single copy of the *T11* mutation. In addition, larvae homozygous for the parent chromosome (*cn bw sp*) show no electrophysiological defects. These results strongly suggest that syt in these larvae is a key component for synaptic vesicle release evoked by Ca^{2+}, but is not required for vesicle fusion through the Ca^{2+}-independent spontaneous MEJP pathway.

Discussion

Two recent reports (DiAntonio et al., 1993; Nonet et al., 1993) have shown that neural transmission in mutants that lack syt is severely affected but persists. DiAntonio et al. (1993) proposed an accessory role for syt in neurotransmission based on the observation that a significant portion of homozygous null embryos hatch and that these embryos exhibit muscle depolarizations suggestive of neurally mediated neurotransmission. We have also investigated the role of syt by characterizing the phenotype of another set of mutations in *syt*. By combining P element–mediated insertion mutagenesis strategies and chemical mutagenesis with EMS, we recovered a series of recessive loss-of-function alleles that range from null alleles lacking the entire coding sequence of *syt* to weak partial loss-of-function alleles (Table 1). Based on our observation of the failure of most homozygous null mutant embryos to hatch,

Table 2. Summary of Electrophysiological Defects in *syt* Mutants

Larva	Ca^{2+} Concentration	EJP Amplitude (mV ± SEM)	MEJP Frequency MEJP/s ± SEM)	MEJP Amplitude (mV ± SEM)	n
Wild type	0.4 mM	13.1 ± 1.6	0.9 ± 0.3	0.8 ± 0.1	4
syt		0.3 ± 0.2	2.8 ± 0.9	0.6 ± 0.1	5
Wild type	1.0 mM	22.3 ± 0.6	2.3 ± 0.8	0.7 ± 0.1	4
syt		1.5 ± 0.7	3.1 ± 0.7	0.7 ± 0.1	4
Wild type	1.8 mM	25.5 ± 1.2	1.2 ± 0.2	0.6 ± 0.1	4
syt		6.3 ± 0.9	3.2 ± 0.8	0.8 ± 0.1	5

Control wild-type larvae were of the genotype *T11/Gla Bc*. Data from mutant larvae represent combined measurements from *T11/T77* and *T11/T11*.

of the severe reduction in number and size of muscle contractions in such embryos, and of the severe defects in the Ca^{2+} activation of evoked neurotransmission in hypomorphic mutant larvae, we propose that syt is essential for Ca^{2+}-activated neurotransmitter release.

Of homozygous or transheterozygous embryos that carry null alleles *Df(2L)N6* or *Df(2L)N19*, 100% fail to hatch 48 hr after egg laying. These embryos exhibit a 5-fold reduction in the number of muscle propagation waves when compared with wild type. In addition, these waves are quite abnormal and much reduced in intensity. When these embryos are manually excised from the chorion and vitelline membranes, they cannot crawl or feed. However, these deficiencies may affect genes other than *syt*, and germline transformation with a *syt* transgene will be required to address this issue specifically.

Approximately 80% of embryos that carry combinations of point mutations that are probably null alleles (e.g., *T7* and *T41*) fail to hatch. These embryos exhibit a 4-fold reduction in muscle propagation waves. First instar larvae that hatch are barely motile, whereas those that are excised from the chorion and vitelline membranes are immobile and exhibit uncoordinated twitching. Although no protein was observed in *T7/Df(2L)C144* embryos using immunocytochemical methods, it is possible that very low levels of syt activity may allow a portion of the embryos to hatch. Based on this analysis of muscle contractions in null and severe hypomorphic embryos, we conclude that syt is required for normal larval muscular contractions. The absence of syt probably leads to embryonic lethality because the severe reduction in number and in coordination of contractions is insufficient for hatching. However, null mutants lacking the entire *syt* coding sequence show some coordinated contractions, even though their number is greatly reduced, suggesting the persistence of some neurotransmission. To determine the cause of the defects in neurotransmission in *syt* mutants, we focused on the electrophysiological characterization of neurotransmission in hypomorphic third instar larval mutants.

The recovery of point mutations that cause a partial lack of function of syt has allowed us to assess the electrophysiological consequences of reduced syt activity at the larval neuromuscular junction (Jan and Jan, 1976). At a low Ca^{2+} concentration (0.4 mM), there appears to be an almost complete block of neurotransmission (see Table 2; see Figure 6). This block can be partially rescued by increasing the extracellular Ca^{2+} concentration to 1 mM, at which concentration we observe a 15-fold reduction in neurotransmission. At a Ca^{2+} concentration of 1.8 mM, there is still a 4-fold reduction in evoked response. This strongly reduced release combined with the facilitation that we have observed may account for the viability of these behaviorally defective third instar larvae. We suggest that the release at high Ca^{2+} concentration is due to partial function of the *T11* allele as suggested by the complementation data and phenotypic analyses. Since there is only one known *syt* gene in Drosophila (DiAntonio et al., 1993; Littleton et al., 1993) that is expressed in central and peripheral nervous system neurons (Littleton et al., 1993), we expect that these defects exist at most or all synapses in these mutants, including synapses in the central nervous system. Given the severity of defects of Ca^{2+}-dependent evoked neurotransmitter release in these partial lack-of-function mutants, we expect that null mutant embryos may not or may very poorly respond to Ca^{2+}-dependent evoked stimuli. Although the interpretations of our data differ from that of DiAntonio et al. (1993), we believe that both sets of data fit the hypothesis that there is a very severe reduction in evoked neurotransmitter release since DiAntonio et al. (1993) recorded greatly reduced spontaneous muscle potentials in null mutant embryos that were not shown to be Ca^{2+} dependent or independent.

We have also observed a 2- to 3-fold increase in MEJP frequency in hypomorphic mutant larvae, suggesting that different mechanisms of spontaneous and evoked neurotransmitter release exist. MEJP frequencies are constant for the three tested Ca^{2+} concentrations, while evoked release is increasingly rescued by a higher Ca^{2+} concentration. In the absence of syt, the MEJPs are not reduced. At a low Ca^{2+} concentration, when evoked release is absent, spontaneous release continues and even shows an increase in frequency. These data support the presence of different mechanisms for the two forms of release and also support the idea that syt is intimately connected with Ca^{2+} activation of evoked neurotransmitter release.

Syt has been shown to bind Ca^{2+} in the presence of phospholipids at concentrations thought to be present at the active zone during neurotransmitter release (Brose et al., 1992). In addition, each syt monomer appears to bind

multiple Ca^{2+}s, consistent with the higher order dependence of neurotransmitter release upon external Ca^{2+} concentration (Dodge and Rahaminoff, 1967). Since our electrophysiological analysis at low Ca^{2+} concentration shows that syt must be present to initiate evoked release, we propose that syt is the major Ca^{2+} sensor for evoked neurotransmission.

Upon activation of syt, two alternative fusion pathways can be envisaged. In the first model, we propose that syt is the synaptic vesicle fusion protein itself. In this model, the C2 domains of syt not only sense Ca^{2+}, but mediate fusion by binding phospholipids and destabilizing closely apposed synaptic vesicle and presynaptic membranes. This action may require an interaction of syt with the presynaptic α-latrotoxin receptor, a member of the neurexin family (Petrenko et al., 1991; Ushkaryov et al., 1992). This hypothesis is supported by the observation that latrotoxin causes massive exocytosis in the absence of Ca^{2+}, possibly by altering the conformation of syt and mimicking Ca^{2+} binding. This model has the advantage that it integrates a role for Ca^{2+} activation of phospholipid binding. It also requires fewer steps, an important consideration given that interactions leading to fusion probably need to occur in the time scale of 200 μs. In the second model, we propose that Ca^{2+} sensing by syt leads to activation of a separate fusion protein or complex of proteins. The second model attempts to integrate observations made on other proteins proposed to be involved in neurotransmitter release, like Synaptobrevin (Link et al., 1993; Schiavo et al., 1992), syntaxins (Bennett et al., 1992), and other synaptic vesicle–associated proteins like SNAP25 (Söllner et al., 1993; Whiteheart et al., 1993) in mediating fusion. In this model, Synaptobrevin, syntaxin, and SNAPs form a docking–fusion complex (Söllner et al., 1993; Whiteheart et al., 1993) whose activity is critically dependent on the action, interaction, or both of syt. Activation of this complex by syt allows fusion to proceed.

Either model could accommodate an inhibitory role of syt on fusion in the absence of Ca^{2+} activation (Popov and Poo, 1993). This inhibitory role may explain the 2- to 3-fold increase in MEJP frequency that we observe in our hypomorphic mutants, as well as the presence of multiple uncoordinated spontaneous muscle contractions observed in mutants that lack syt. Since MEJPs exist in neuromuscular junctions of hypomorphic mutants and since they probably result from the fusion of docked vesicles, these mutants are probably not defective in docking. Resolution of the role of syt in docking will require careful electron microscopy analysis of synapses in *syt* null mutants to determine whether there are any discernible changes in the number or appearance of docked vesicles.

The obvious differences in effect of *syt* mutations on spontaneous and evoked release indicate that multiple mechanisms exist for neurotransmitter release. If one adds an additional mechanism for the neurosecretion of large dense core vesicles, it is possible to understand the variety of interpretations for the role of syt in neurotransmitter release in different systems. Shoji-Kasai et al. (1992) have shown that uninduced PC12 cells selected to be deficient for syt can release dopamine and ATP. This release of neurotransmitter may reflect a separate fusion pathway present in secretory cells. Elferink et al. (1993) showed that intracellular injection of antibodies against syt into PC12 cells induced with nerve growth factor inhibit neurotransmitter release as measured by assaying extracellular exposure of dopamine-β-hydroxylase, an enzyme of some synaptic and secretory vesicles, suggesting that syt is critical for their release. These results may reflect that, after nerve growth factor induction, PC12 cells release neurotransmitter via a pathway that is more similar to the fusion of synaptic vesicles at synapses. Electrophysiological recordings of the squid giant synapse, where presynaptic injections of peptides homologous to the C2 domain of syt block neurotransmission (Bommert et al., 1993), support our interpretation because this study focuses on Ca^{2+}-evoked release from synaptic vesicles. Finally, concurrent work in Drosophila (DiAntonio et al., 1993; this work) and C. elegans (Nonet et al., 1993) demonstrates that continued but greatly reduced release in null mutants may result from Ca^{2+}-independent release or a default secretory pathway that persists in the absence of syt. Such a pathway has been shown to be present in multiple nonneuronal cell types (Walch-Solimena et al., 1993). Further characterization of the now-growing collection of null and loss-of-function mutations, as well as localization of the structural defects in syt, should provide clues into how syt functions in Ca^{2+}-activated vesicle fusion and synapse function not only at the neuromuscular junction but potentially at all synapses.

Experimental Procedures

Molecular Techniques

Southern analysis and chromosomal walking were performed as described by Sambrook et al. (1989). Exon–intron boundaries were mapped by Southern hybridization. Genomic sequences that hybridize to the pD65-1 cDNA were subcloned into pBluescript (Stratagene) and partially sequenced. This allowed determination of the precise exon–intron boundaries of all exons shown, except a portion of exon 2 that may consist of two exons. Sequencing methodologies are described in Bellen et al. (1992). DNA sequence analysis was carried out by the Nucleic Acids Core in the Institute for Molecular Genetics at Baylor College of Medicine.

Fly Strains and Mutagensis

Flies were cultured on standard fly food supplemented with dry yeast. Strains *Df(2L)DTD2 Sp Bl* (22D4-5;23B1-2) *Dp(2;2)dpp^{d21}/Gla Bc* (abbreviated *Df(2L)DTD2*) and *dpp$^{d\text{-}ho}$Df(2L)C144 ed (23A1-2;23C)/Gla Bc Elp* (abbreviated *Df(2L)C144*) and *dpp^{hr4} 66.4/Gla* (abbreviated *66.4*) were obtained from J. Sekelsky and W. Gelbart. The *y w*;P[*lacZ,w*]B8-2-30 (abbreviated *B8*) strain was obtained from G. Feger and Y. N. Jan. The *cn bw sp* flies were obtained from the Indiana Stock Center at Bloomington.

The homozygous viable P[*lacZ,w*]B8 (Bier et al., 1989) insertion chromosome was isogenized and then mobilized essentially as described by Tower et al. (1993). Flies with eye colors differing from those of heterozygous *B8* flies were selected as they probably contain insertions at novel positions. Using the primers shown in Figure 2, 3200 flies were screened. The location of the *T77* insertion was confirmed by both PCR and Southern analysis (see Figure 2). The original viable *B8* insertion is still present in the *T77* chromosome, as is often observed when local hops occur (Tower et al., 1993). However, genomic Southern analysis indicates that the *B8* insertion is unaffected in the *T77* chromosome. Wild-type revertant *N1* was generated by reintroducing the Δ2-3 transposase into the *T77* genetic background. *N1* flies still carry the *B8* insertion.

Excisions *N6* and *N19* were generated by reintroducing the Δ2-3 transposase into the *T77* flies. The excision strains were examined with PCR using primers 5′ and 3′ to the original insertion and by Southern analysis. *Df(2L)N6* and *Df(2L)N19* do not affect the 5′ region of the *T77* P element, yet they remove the 3′ portion of the insertion as well as the *syt* locus. The *B8* insertion is not affected in *Df(2L)N6* chromosomes and is excised in *Df(2L)N19*.

Novel alleles of *syt* were generated by feeding isogenized adult *cn bw sp/cn bw sp* males EMS as previously described (Lewis and Bacher, 1968). These males were crossed to *Df(2L)DTD2/Gla* females (*Gla* is an inversion that balances the left arm of the second chromosome). Individual *cn bw sp*/Gla* males were crossed to *Df(2L)DTD2/Gla* females. Chromosomes that fail to complement *Df(2L)DTD2* were recovered to establish *cn bw sp*/Gla* strains. These chromosomes were subsequently tested over the *T77* and *Df(2L)N6* chromosomes. Failure to complement the *T77* insertion and *Df(2L)N6* indicates that they contain mutations in *syt*. This was further confirmed by other complementation tests.

Whole-Mount Embryo Procedures

Digoxigenin-labeled probes were prepared as described in the DNA labeling and detection kit (nonradioactive) from Boehringer Mannheim. In situ hybridizations to whole-mount embryos were performed essentially as previously described (Tautz and Pfeifle, 1989). X-Gal stainings of whole-mount embryos were performed as described by Bellen et al. (1989). Immunocytochemical staining of whole-mount embryos is as described by Littleton et al. (1993).

Phenotypic Analyses

Peristaltic contractions were counted in 21–22 hr wild-type and mutant embryos submersed in halothane oil to visualize the embryonic cuticle. Embryos lacking syt were identified as those that failed to hatch 48 hr after egg laying. Heterozygous flies were first outcrossed to Canton-S flies, and heterozygous flies carrying different *syt* alleles were crossed to each other and allowed to lay eggs for 2 hr.

Electrophysiology

Third instar larvae for electrophysiological analysis were grown at 25°C in uncrowded bottles or vials. Wild-type larvae were collected from a stock of *T11/Gla Bc*. Larvae transheterozygous for *T11* and *T77* were produced in the following manner. Fly strains were constructed that are heterozygous for either *T11* or *T77* and a chromosome carrying a translocation between the second chromosome balancer *SM5* and the third chromosome balancer *TM6B-Tubby*. These two lines were crossed together, and non-*Tubby* larvae, which are transheterozygous for *T11* and *T77*, were selected for analysis. Larvae that were homozygous for *T11* were produced in a similar manner. Dissections, nerve stimulation, and muscle recordings were performed as described previously (Ganetzky and Wu, 1982; Stern and Ganetzky, 1989). Nerves innervating the body wall muscles were cut near the ventral ganglion and were stimulated with a suction electrode. For intracellular muscle recordings, microelectrodes were pulled on a Flaming–Brown micropipette puller to tip resistances of about 20 MΩ and were filled with 3 M KCl. Nerves were stimulated for 0.1 ms at a voltage of 1.5 times threshold voltage. Muscle cell 6 or 7 from abdominal segments 4 or 5 were used for data collection. All dissections and recordings were performed at 21°C–22°C. Data were digitized at 5–25 kHz and analyzed with the MacAdios II and Superscope systems from G. W. Instruments.

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