Cell, Vol. 73, 1281–1290, July 2, 1993, Copyright © 1993 by Cell Press

Synaptic Transmission Persists in *synaptotagmin* Mutants of Drosophila

Aaron DiAntonio, Karen D. Parfitt, and Thomas L. Schwarz
Department of Molecular and Cellular Physiology
Stanford University Medical Center
Stanford, California 94305-5426

Summary

Synaptotagmin is one of the major integral membrane proteins of synaptic vesicles. It has been postulated to dock vesicles to their release sites, to act as the Ca^{2+} sensor for the release process, and to be a fusion protein during exocytosis. To clarify the function of this protein, we have undertaken a genetic analysis of the synaptotagmin gene in Drosophila. We have identified five lethal alleles of *synaptotagmin*, at least one of which lacks detectable protein. Surprisingly, however, many embryos homozygous for this null allele hatch and, as larvae, crawl, feed, and respond to stimuli. Electrophysiological recordings in embryonic cultures confirmed that synaptic transmission persists in the null allele. Therefore, *synaptotagmin* is not absolutely required for the regulated exocytosis of synaptic vesicles. The lethality of *synaptotagmin* in late first instar larvae is probably due to a perturbation of transmission that leaves the main apparatus for vesicle docking and fusion intact.

Introduction

Release of neurotransmitter from chemical synapses is the primary method of communication between neurons, and the regulation of this process is crucial for plastic changes within the nervous system (Jessell and Kandel, 1993). Neurotransmitter is stored in synaptic vesicles that must fuse with the presynaptic membrane so that transmitter can be released into the synaptic cleft and diffuse to the postsynaptic cell. This process has much in common with other membrane trafficking events, such as Golgi transport, including the probable participation of small GTP-binding proteins (Bourne et al., 1990). One fundamental difference, however, is the tight regulation by intracellular Ca^{2+} of the fusion of synaptic vesicles with the plasma membrane. Ca^{2+} entry through voltage-dependent Ca^{2+} channels couples the electrical impulse in the cell to the release of vesicles. A second fundamental difference is the speed of the process. The delay between the arrival of the action potential and the release of neurotransmitter can be as short as 0.2 ms. This rapid time course and the morphology of the terminal as determined by electron microscopy (Couteaux and Pecot-Dechavassine, 1973) indicate that a releasable population of vesicles must be docked at the presynaptic membrane in a fusion-ready state. Though many of the protein constituents of synaptic vesicles have been sequenced and characterized, the biochemical mechanisms of docking, fusion, and regulation by Ca^{2+} remain enigmatic.

Vesicle-associated proteins are likely to be crucial to these processes. One such protein, synaptotagmin (p65), has been extensively studied. This membrane protein of approximately 65 kd is found only in the nervous system and certain endocrine secretory cells. It is in the membranes of small clear vesicles, such as those found at most synapses, and also in chromaffin granules (Wendland et al., 1991; Perin et al., 1991b). Synaptotagmin has been cloned from rat, electric fish, human, and Drosophila (Perin et al., 1990; Wendland et al., 1991; Perin et al., 1991a). The protein consists of a short N-terminal intravesicular tail, a single transmembrane domain, and a large cytoplasmic domain with two repeats that have homology to the C2 regulatory domain of protein kinase C (PKC). This motif has also been identified in an isoform of phospholipase A2 (Clark et al., 1991) and in proteins that interact with small GTP-binding proteins (Vogel et al., 1988, Shirataki et al., 1993). The phospholipase A2 and PKC isoforms that include this motif translocate from a cytoplasmic pool to a membrane-associated pool when stimulated. It is within these C2 repeats that synaptotagmin shows the strongest cross-species homology. In Drosophila the first and second repeats are 78% identical to their homologs in rat, although they are only 44% identical to each other. In rats two closely related isoforms of synaptotagmin have been identified (Geppert et al., 1991); two similar isoforms (p65-A and p65-B) have been identified in Discopyge ommata, along with a third more divergent form (p65-C) (Wendland et al., 1991). Drosophila appears to have only a single isoform (see below). Several other proteins implicated in transmitter release have been found to have homologs in nonneuronal cells, including yeast, and may therefore have functions that are also necessary in other types of membrane trafficking (Gerst et al., 1992; Segev et al., 1988; Salminen and Novick, 1987; Hardwick and Pelham, 1992). Synaptotagmin, at present, has not been found outside the nervous and endocrine systems and may therefore have a function that is unique to the regulated secretory pathway (Wendland et al., 1991).

Recent biochemical experiments have suggested that synaptotagmin may play a central role in synaptic vesicle physiology. Based on its sequence and demonstrated lipid-binding capabilities, Perin et al. (1990) have suggested that synaptotagmin may mediate membrane interactions during synaptic vesicle exocytosis, i.e., that it may be a fusion protein. Bennett et al. (1992) have demonstrated that synaptotagmin binds to syntaxin, a molecule present in the presynaptic membrane. Syntaxin in turn associates with the N-type Ca^{2+} channel. This led them to propose a model in which synaptotagmin docks vesicles to release sites by binding a syntaxin–Ca^{2+} channel complex. This interaction could have implications for the etiology of Lambert–Eaton myasthenic syndrome (Leveque et al., 1992). Synaptotagmin also binds to the α-latrotoxin

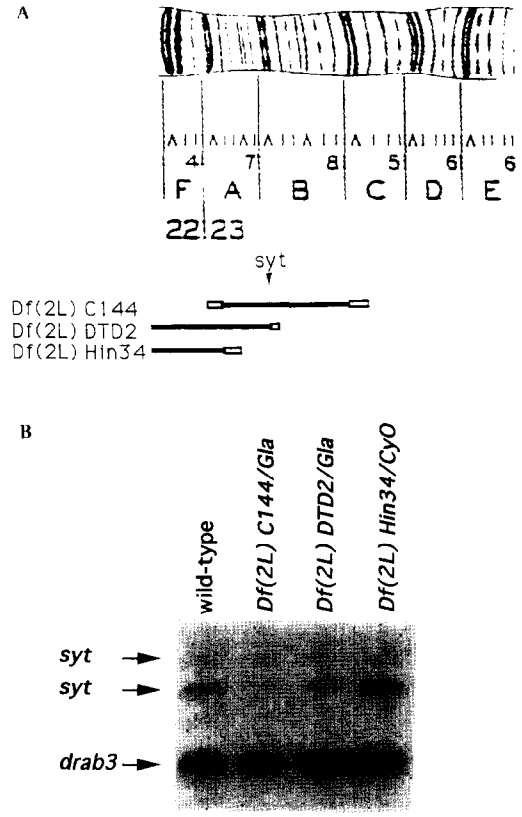

Figure 1. The Location of the *syt* Gene and Deficiencies in the Region

(A) The region between 22F and 23D of the left arm of the second chromosome is shown, with the location of the *syt* gene indicated. Three chromosomal deficiencies were identified that remove either the *syt* gene or more distal portions of the chromosome. The absent regions are indicated by horizontal lines. Open boxes represent uncertainty in the assignment of endpoints to these deficiencies. The polytene chromosome map is taken from Sorsa (1988).

(B) The presence of the *syt* gene within the boundaries of two deficiencies was shown by quantitative genomic Southern blot. Genomic DNA was prepared from wild-type *Oregon R* (*OrR*), *Df(2L)C144/Gla*, *Df (2L) DTD2/Gla*, and *Df(2L)Hin34/CyO* flies and digested with EcoRI. The DNA was subjected to Southern blot analysis, and the blot was probed with a *syt* open reading frame probe and, as a control for the quantity of DNA loaded per lane, a *drab3* open reading frame probe. In those deficiencies that remove *syt*, the gene is present only on the balancer chromosomes, and the *syt* signal is half the strength of the signal from wild-type DNA. The *syt* signal is clearly weaker in the *Df(2L)C144/Gla* and *Df(2L)DTD2/Gla*, demonstrating that these two deficiencies remove *syt* while *Df(2L)Hin34* does not.

receptor (Petrenko et al., 1991) and to the intracellular receptors for protein kinase C (Mochly-Rosen et al., 1992). Finally, synaptotagmin has been implicated as the Ca^{2+} sensor for synaptic vesicles. Brose et al. (1992) have demonstrated that purified synaptotagmin is capable of binding both acidic phospholipids and Ca^{2+}. They suggest that Ca^{2+} triggers a conformational change in synaptotagmin such that its cytoplasmic domains interact with phospholipids in the plasma membrane and initiate vesicle fusion. While these biochemical experiments suggest that synaptotagmin is a fusion, docking, and/or Ca^{2+}-sensing protein, a recent experiment in PC12 cells found no requirement for synaptotagmin in Ca^{2+}-dependent neurotransmitter release. Shoji-Kasai et al. (1992) created a mutant PC12 cell line that did not express synaptotagmin but that still released transmitter from dense-core vesicles when measured over a period of minutes. However, the injection of antibodies to synaptotagmin or cytosolic fragments of the protein has been observed to decrease exocytosis from PC12 cells (Elferink et al., 1993). Thus, the function of synaptotagmin remains an open question.

To address this question, we have undertaken a genetic analysis in Drosophila. To date only one synaptotagmin homolog has been identified in Drosophila, despite extensive searches by our lab and others. This gene is expressed throughout the nervous system in embryos, larvae, and adults. An antiserum to the cytoplasmic domain of rat synaptotagmin intensely stains synaptic regions of the Drosophila central nervous system and synaptic boutons at neuromuscular junctions (DiAntonio et al., submitted). If synaptotagmin plays a central role in vesicle fusion, docking, or Ca^{2+} sensing, then one might predict that a null mutant for this protein would profoundly disrupt synaptic transmission. In this study we report the identification of 5 mutant alleles of *synaptotagmin* (*syt*), including one that lacks detectable syt protein. All of these mutants are lethal, demonstrating that *syt* is an essential gene in Drosophila. However, all of these mutants exhibit complex behaviors before dying: they crawl, eat, and respond to sensory stimulation. In addition, spontaneous excitatory junctional currents were recorded in cultures of homozygous null *syt* mutant embryos. This demonstrates that synaptic transmission still occurs in the absence of the syt protein. A similar observation in Caenorhabditis elegans is reported in the accompanying paper by Nonet et al. (1993 [this issue of *Cell*]).

Results

Isolation of *syt* Mutations

The Drosophila *syt* gene was localized to position 23B on the left arm of chromosome 2 by in situ hybridization of a cDNA clone to polytene chromosomes. To isolate mutations in this gene, chromosomal deficiencies that remove *syt* were identified (Figure 1A). The presence of *syt* within the boundaries of a deficiency was determined either by in situ hybridization to polytene chromosomes or by quantitative analysis of genomic Southern blots with the *drab3* gene as an internal standard (Figure 1B). The *syt* gene is removed by *Df(2L)C144* and *Df(2L)DTD2* but not by *Df(2L) Hin34*. The cytological boundaries of these deficiencies are indicated in Figure 1. This deficiency mapping locates *syt* between 23A2–4 and 23B1–2, in agreement with the mapping by in situ hybridization. The *Curly* (*Cy*) gene, a commonly used dominant marker, is also present in this region.

To isolate mutations in *syt*, we screened for recessive lethal mutations that fell in the region deleted by *Df(2L) C144*. Ethyl methanesulfonate–mutagenized second chromosomes (8000) were tested in an F2 screen (see Experimental Procedures), and 85 mutants were identified. We could rule out 53 of these as potential *syt* mutations, be-

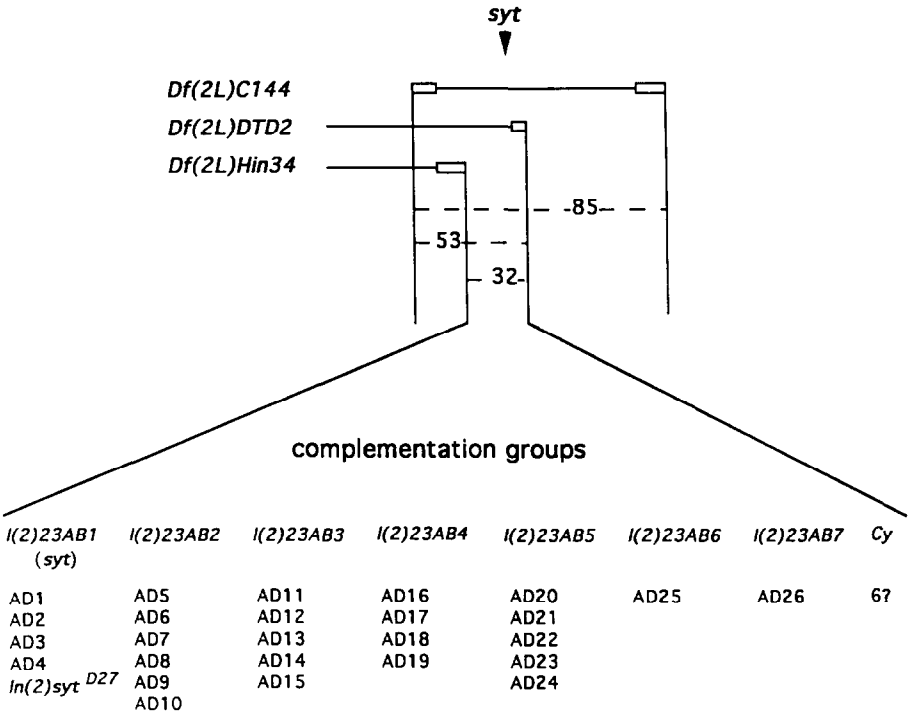

Figure 2. F2 Screen for Lethals under *Df(2L)C144*

An F2 lethal screen identified 85 mutants that are lethal in combination with *Df(2L)C144*. Thirty-two mutants were designated as potential *syt* alleles, because they were lethal in combination with *Df(2L)DTD2* but viable with *Df(2L)Hin34*. Most of these mutants were placed into 1 of 7 complementation groups designated *l(2)23AB1* through *l(2)23AB7*, though we have not established their order in the genome. Each allele is named with the superscript AD followed by a number. The *l(2)23AB1* complementation group was subsequently shown to be the *syt* gene by genetic and molecular criteria. A homozygous lethal chromosomal rearrangement that we have called *ln(2)syt^{D27}* was also placed in the *l(2)23AB1* complementation group. The gene for the commonly used Drosophila marker *Cy* is also present within this region, and a number of mutants were identified that showed some form of interaction with previously described *Cy* alleles.

cause they were not lethal in combination with *Df(2L)DTD2* or were lethal in combination with *Df(2L) Hin34*. The remaining 32 mutants that showed the complementation pattern expected for *syt* were placed into 1 of 8 complementation groups by classical complementation testing. One group genetically interacted with *Cy*, and its members are tentatively designated as new alleles of *Cy*. Of the other 7 lethal complementation groups identified, 5 included 4 to 6 alleles each, while the other 2 were represented by a single allele (Figure 2). We have named these complementation groups *l(2)23AB1* through *l(2)23AB7*. In the experiments that follow, we will provide evidence that one of these, *l(2)23AB1*, is *syt*.

We have identified a γ-ray-induced rearrangement in *syt* that has proven very useful both in identifying *l(2)23AB1* as the *syt* complementation group and in analyzing the phenotype of *syt*. This rearrangement, which we are calling *ln(2)syt^{D27}*, was generously provided by J. Sekelsky and W. Gelbart, and its associated lethality maps to the same interval as *syt*; the rearranged chromosome fails to complement *Df(2L)DTD2* and *Df(2L)C144* but does complement *Df(2L)Hin34*. When tested against the ethyl methanesulfonate–induced lethals, *ln(2)syt^{D27}* failed to complement the 4 *l(2)23AB1* alleles but did complement all alleles of the other 7 complementation groups. Thus, the lethality of *ln(2)syt^{D27}* in combination with *Df(2L)C144* is probably due to the disruption of a single gene, *l(2)23AB1*.

Genetic evidence that *l(2)23AB1* and *ln(2)syt^{D27}* are mutations of *syt* was obtained by rescuing them with a minigene. The *syt* gene itself is large, covering more than 20 kb of genomic DNA (DiAntonio et al., submitted). Since transformation frequency decreases with increasing construct size, we chose to rescue instead with a minigene construct. A P transposable element was constructed that carries a 2.2 kb cDNA that includes the entire open reading frame of *syt*. Because the *syt* promoter has not been identified, the expression of the cDNA was driven by the *elav* promoter, a well-characterized Drosophila promoter that is active in all neurons (Robinow and White, 1988). This promoter was chosen because the expression pattern of *syt* is qualitatively similar to that of *elav*. The P element, referred to as P[elav-syt], was introduced into embryos, and a transformant with an insertion onto the third chromosome was obtained. Flies hemizygous for *ln(2)syt^{D27}* or 1 of the 4 ethyl methanesulfonate alleles of the *l(2)23AB1* complementation group (i.e., flies containing the mutant chromosome placed over a deficiency chromosome) were viable in the presence of P[elav-syt]. Although the lethality is rescued, the rescued flies are clearly abnormal. Their walking is sluggish and uncoordinated, and most are un-

Figure 3. The *In(2)syt*^{D27} Mutation Has a Breakpoint at *syt*

A genomic *syt* clone was hybridized to the polytene chromosomes of a heterozygous *In(2)syt*^{D27} larva. Signals, indicated by arrows, are detected as a single strong band on the wild-type chromosome and two fainter bands on the rearranged chromosome. Thus, the *In(2)syt*^{D27} rearrangement appears to have a breakpoint within the *syt* gene.

able to fly. With the exception of *l(2)23AB1*^{AD3}, which may contain a second lethal mutation at an unrelated site on the chromosome, P[elav-syt] also rescues the homozygous lethality of all the alleles in this complementation group. None of the mutations in the other *l(2)23AB* genes could be rescued by P[elav-syt]. These data constitute genetic proof that the rescued mutants are *syt* alleles. The *l(2) 23AB1* complementation group will be called *l(2)syt*.

A Null Allele of *syt*

The nature of the *In(2)syt*^{D27} mutation was examined by probing salivary gland chromosomes from *In(2)syt*^{D27}/+ larvae with a 30 kb piece of genomic DNA from the *syt* gene. The probe hybridizes to a single band at 23B on the wild-type chromosome but hybridizes to two locations on the *In(2)syt*^{D27} chromosome (Figure 3). Hence, *In(2)syt*^{D27} appears to be an inversion with a breakpoint in the *syt* gene.

To define the molecular location of the *In(2)syt*^{D27} breakpoint, digests of genomic DNA from *In(2)syt*^{D27} mutants were compared with DNA from flies that carried the parent chromosome, *DTD52*, on which the *In(2)syt*^{D27} had been induced, and another related chromosome, *DTD67* (Figure 4A). Following digestion with each of four enzymes, the DNA was analyzed on Southern blots by hybridization to a probe made from the region of the gene that encodes amino acids 94 to 448 (probe A). With each enzyme, it is apparent that *In(2)syt*^{D27} exhibits new bands. The pattern with EcoRI is more complex because of a phenotypically silent polymorphism (an additional EcoRI site) that distinguishes the *DTD52* chromosome and its derivatives from either wild-type (*OrR*) or balancer (*CyO*) chromosomes. This polymorphism converts a 4.8 kb EcoRI fragment into two fragments of 1.0 and 3.8 kb. It is this 3.8 kb EcoRI fragment, a fragment in the middle of the *syt* transcription unit (DiAntonio et al., submitted), that is disrupted by the *In(2)syt*^{D27} breakpoint (Figure 4A, lane B, EcoRI). When digested with Bsp106, both polymorphic fragments generated by the breakpoint hybridize to this probe and are detectable on the Southern blot (Figure 4A, arrows). Since this probe hybridizes to DNA on both sides of the breakpoint, the breakpoint must occur within the limits of this probe. To define more precisely the location of the breakpoint, shorter probes (probes B and C) were tested, and each was found to hybridize to both polymorphic bands (Figure 4A, arrows) and therefore to span the breakpoint. This places the breakpoint in the overlap between probes B and C, which corresponds to that portion of the gene encoding amino acids 303 to 350. This placement was further refined by sequencing of genomic DNA; the sequence from amino acid 322 onward was not in the 3.8 kb EcoRI fragment disrupted by the breakpoint. Therefore, the breakpoint must fall between amino acids 303 and 322. This part of the protein is the linker between the first and second PKC homology domains. Hence, any syt protein expressed in *In(2)syt*^{D27} homozygotes would be severely truncated, lacking the entire second C2 domain (Figure 4B).

To assess the possibility that this truncated protein is expressed in *In(2)syt*^{D27}, we used a rabbit polyclonal antiserum raised to the cytoplasmic domain of rat synaptotagmin. We have previously demonstrated in wild-type Drosophila that this serum stains the neuropil of embryos and adults and larval neuromuscular junctions (DiAntonio et al., submitted). The major epitopes recognized by this serum reside in the first PKC C2 domain: the serum preferentially recognizes the first repeat of rat synaptotagmin (B. Wendland, personal communication), and that repeat is necessary in preincubations for blocking staining of Drosophila synapses (data not shown). Thus the breakpoint, as localized above, falls distal to the major epitopes, and a protein truncated at the breakpoint would be detected by this serum. The *In(2)syt*^{D27} mutation is kept as a balanced stock over a chromosome marked with a β-galactosidase gene, so that homozygous embryos can be identified by the absence of β-galactosidase staining. Homozygous *In (2)syt*^{D27} embryos that were identified in this manner and stained with the anti-synaptotagmin antibody showed no detectable immunoreactivity in the nervous system (data not shown; see Figure 5). Therefore, embryos genetically identified as *In(2)syt*^{D27} homozygotes are deficient for the protein recognized by this antiserum. Thus, if any protein is produced in *In(2)syt*^{D27} homozygotes, it must be both severely truncated and so rare as to be undetectable with the antiserum. *In(2)syt*^{D27} appears to be a null allele of *syt*.

Synaptic Transmission Persists in a *syt* Null

All of the *syt* mutations are hemizygous lethal, including the putative null *In(2)syt*^{D27}, so *syt* must be considered an essential gene in Drosophila. *In(2)syt*^{D27} homozygotes die as late embryos and first instar larvae. When *In(2)syt*^{D27}/+ adults are crossed to +/+ adults and embryos are collected, all embryos hatch within 24 hr at 24°C. Thus, het-

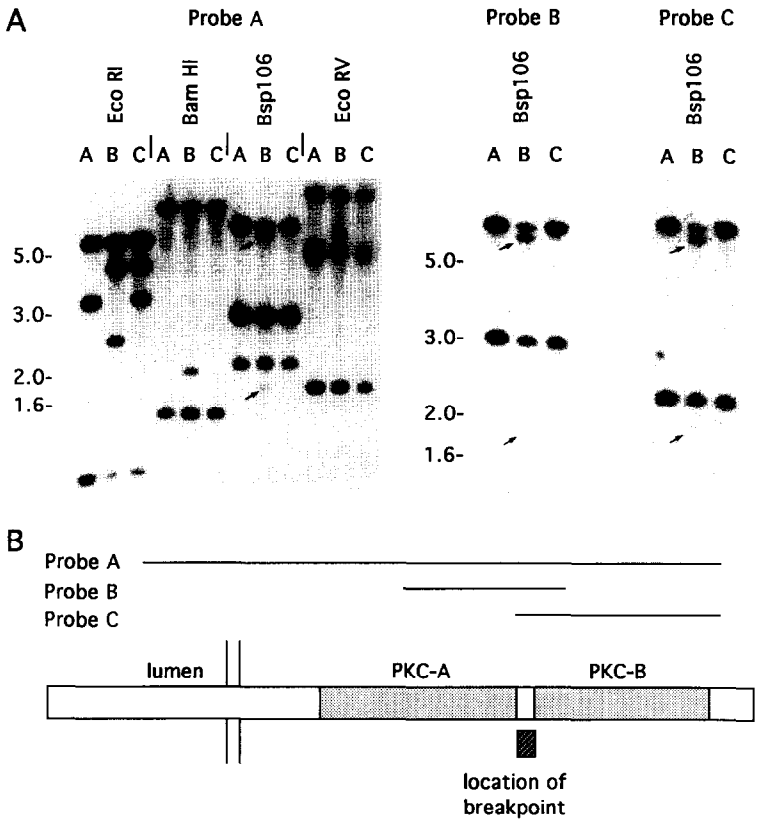

Figure 4. The *In(2)syt*^{D27} Mutation Disrupts the Open Reading Frame of *syt*

(A) Southern blot analysis was performed on genomic DNA from *DTD52* (lane A), which is the parental strain for *In(2)syt*^{D27}, *In(2)syt*^{D27}/*CyO* (lane B), and another related chromosome, *DTD67/CyO* (lane C). DNA was digested with EcoRI, BamHI, Bsp106, or EcoRV, as indicated. When probed with probe A, corresponding to that part of the gene that encodes amino acids 94–448, polymorphic bands were apparent in each of the digestions of *In(2)syt*^{D27} DNA. To define the location of the breakpoint more precisely, a similar blot was probed with probe B (corresponding to amino acids 237–350) and probe C (corresponding to amino acids 303–448). Since all three probes hybridize to both polymorphic bands (arrows) in the DNA digested with Bsp106, the breakpoint must be located within the overlap of these probes. The location of the breakpoint was further refined by genomic sequencing (see Results).

(B) A cartoon of the synaptotagmin protein depicts its intravesicular tail, transmembrane region, and cytoplasmic portion, with the regions of homology to the C2 domains of PKC stippled. The site of the breakpoint in the *In(2)syt*^{D27} mutation is indicated.

erozygotes for *In(2)syt*^{D27} hatch in a normal period of time. When *In(2)syt*^{D27}/+ adults are crossed to each other, about one-quarter of the embryos (the number predicted to be homozygous for the mutation) do not hatch within 24 hr. These embryos appear to be fully developed first instar larvae, yet most do not hatch from their chorion. About one-third of these developmentally delayed larvae are eventually able to emerge, and the remainder move within the egg case but do not hatch (Table 1). Movement by the embryo within the chorion normally occurs prior to hatching and has been shown to derive from synaptic stimulation of the embryonic musculature (Broadie and Bate, 1993). When the delayed, mutant embryos do hatch or are dissected free from their chorion, they are capable of sluggish crawling and feeding behaviors. When reared at 18°C, these delayed larvae are able to live for over a week, but they do not grow or molt as wild-type larvae would. When touched on the head or tail, these larvae attempt to retreat from the stimulus in qualitatively the same manner as wild-type larvae.

To prove that the phenotypically sluggish larvae are indeed *In(2)syt*^{D27} homozygotes, we stained wild-type and putative mutant larvae with the anti-synaptotagmin antiserum. The central nervous system of the wild-type larvae showed intense staining in the synaptic neuropil (Figure 5A), while the putative mutant larvae's central nervous system did not stain (Figure 5B). This confirms the phenotypic identification of *In(2)syt*^{D27} homozygotes. These *In(2)syt*^{D27} homozygotes, lacking detectable syt protein, are capable of performing the complex behaviors described above; synaptic transmission must still occur in these *syt* nulls.

Each of the other 4 *syt* alleles in combination with *In(2)syt*^{D27} produced similar developmentally delayed larvae (Table 1), although certain allelic combinations produced more vigorous larvae that are capable of some growth and maturation. These more vigorous alleles are presumably not null, and preliminary evidence from immunochemistry indicates that some of our *syt* alleles produce protein.

The evidence presented above that *In(2)syt*^{D27} is a null allele comes from a molecular characterization of the mutation and immunocytochemistry. The classical genetic test of a null allele compares the phenotype of the putative null as a homozygote to the phenotype of the null in combination with a deficiency chromosome. We could not perform this test quantitatively, because our deficiency chromosomes remove large regions of the genome and have fertility and developmental abnormalities as heterozygotes. Qualitatively, however, *In(2)syt*^{D27} over deficiency chromosome larvae behave as *In(2)syt*^{D27} homozygotes; most can move, and some are capable of hatching and crawling. To construct an unequivocal null, *Df(2L)DTD2/Df(2L)C144* larvae were generated and assayed for their ability to move. These overlapping deficiencies remove both copies of the entire *syt* gene, in addition to more than eight other lethal genes (see Figure 2). None of these larvae hatch, but when mechanically dechorionated most can move. Some of these larvae have peristaltic body wall

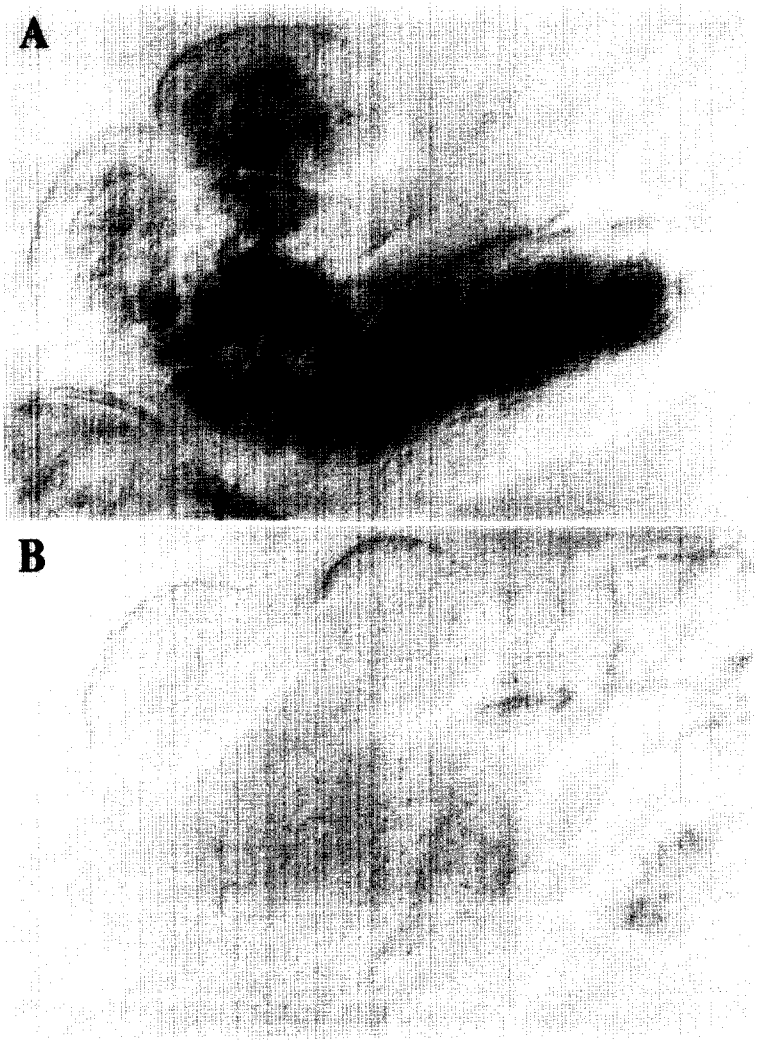

Figure 5. Synaptotagmin Immunoreactivity Is Absent in *In(2)syt*^{D27} Homozygotes

Whole-mount antibody staining with an anti-synaptotagmin antiserum reveals intense staining of the synapse-rich neuropil in the central nervous system of wild-type (A), but not homozygous *In(2)syt*^{D27} (B), first instar larvae. These tissues were dissected from larvae that had successfully hatched from their egg case. The central nervous system of the larva consists of an outer layer of cell bodies (unstained) that surrounds the neuropil.

movements that resemble those in embryos that have been shown to be neurogenic (Broadie and Bate, 1993). Some of these embryos can even move their head and crawl. The genotypic identification of these larvae was confirmed by antibody staining as described above.

In preliminary experiments we have looked directly for synaptic currents in *In(2)syt*^{D27} homozygous embryos (Figure 6). The neuromuscular junctions of dissected whole embryos develop in culture, and these glutaminergic synapses are accessible to whole-cell patch clamp recordings (Broadie and Bate, 1993). Embryos were collected from *In(2)syt*^{D27}*/CyO* parents, and, after recordings were made, the homozygous *syt* mutants could be recognized by β-galactosidase staining (the *CyO* chromosome was marked with *lacZ*; see Experimental Procedures). In each of three mutant embryos, whole-cell recordings from muscle 6 in the body wall revealed spontaneous excitatory junctional currents (*syt*$^{-}$, Figure 6). Similar events were seen in the *syt*$^{+}$ sibling embryos. These events resemble the action potential–dependent excitatory junctional currents reported by Broadie and Bate (1993). Because of the small number of successful recordings, the variability in age of the embryos examined, and the variability of the size of the events in both classes of embryos, we cannot at present make any quantitative comparison of the amplitude, shape, or frequency of these synaptic currents. These observations, however, directly indicate that synaptic transmission persists at a glutaminergic synapse in a mutant lacking synaptotagmin.

Discussion

We have identified 5 mutant alleles of the *syt* gene and have shown that synaptic transmission can persist in the absence of synaptotagmin. This conclusion rests on three key points: first, that we have correctly identified mutations in *syt*; second, that at least one of the mutant lines is truly lacking syt protein; and third, that no closely homologous and potentially redundant second synaptotagmin gene is present at the synapses responsible for the coordinated behavior of the mutants. These points will be addressed here.

Table 1. *syt* Mutants Hatch

Cross	Number Hatched/ Number Total 0–24 hr (%)	After 24 hr (%)
$In(2)syt^{D27}/+ \times In(2)syt^{D27}/+$	73	8[a]
$In(2)syt^{D27}/+ \times +/+$	95	<1
$syt^{AD1}/+ \times In(2)syt^{D27}/+$	75	20
$syt^{AD1}/+ \times +/+$	96	<1
$syt^{AD2}/+ \times In(2)syt^{D27}/+$	77	20
$syt^{AD2}/+ \times +/+$	95	0
$syt^{AD3}/+ \times In(2)syt^{D27}/+$	81	7
$syt^{AD3}/+ \times +/+$	95	<1
$syt^{AD4}/+ \times In(2)syt^{D27}/+$	74	19
$syt^{AD4}/+ \times +/+$	93	<1

Two- to three-hour-old embryos generated from the crosses indicated were placed on a grid on a bed of apple juice–agar, and their ability to hatch was assayed at 24, 36, and 48 hr after egg laying. All 5 *syt* alleles were crossed to both wild-type *OrR* flies and the $In(2)syt^{D27}$ parental stock *DTD52* as a control, and their progeny hatched within 24 hr. This demonstrates that none of the *syt* alleles are developmentally delayed as heterozygotes. However, when the 5 mutants were crossed to the putative *syt* null $In(2)syt^{D27}$, the *syt* mutants still hatched but were developmentally delayed. Although only some of the homozygous $In(2)syt^{D27}$ larvae could hatch, the vast majority moved within their egg shell before dying. The identification of the developmentally delayed progeny of $In(2)syt^{D27}$ heterozygous parents as $In(2)syt^{D27}$ homozygotes was confirmed by antibody staining (see Figure 5). Since these putative nulls for synaptotagmin protein can hatch and perform other complex behaviors, synaptic transmission must persist in the absence of synaptotagmin protein.
[a]After 24 hr, 22% move in egg case.

The identification of 5 mutations in *syt* was demonstrated by both molecular and genetic criteria. Molecularly, one allele ($In(2)syt^{D27}$) was shown to contain a DNA rearrangement in the middle of the *syt* coding region both by analysis of Southern blots and by hybridization to polytene chromosomes. Genetically, all of the alleles were proven to be *syt* mutations by rescue with a P element containing a *syt* minigene.

For an analysis of the null phenotype, we have concentrated on the $In(2)syt^{D27}$ allele. Our identification of $In(2)syt^{D27}$ as a true null is supported by a number of experiments. On Southern blots and by genomic sequencing, we have determined that the $In(2)syt^{D27}$ breakpoint falls within that portion of the open reading frame that encodes amino acids 303 to 322. This part of the protein links the first and second PKC C2 domains. Thus, the most complete protein that could possibly be expressed by the *syt* gene in the $In(2)syt^{D27}$ mutant would lack one of these highly conserved domains. Using an antiserum that recognizes the Drosophila synaptotagmin protein, we have found no evidence for the production of this truncated protein. In embryos genetically identified as homozygous mutants, the neuropil was devoid of detectable protein. Perhaps this is because the lack of the proper polyadenylation signal in the rearranged gene prevents RNA transport from the nucleus or because the truncated protein product is unstable. Therefore, if any protein is produced by the *syt* gene in the $In(2)syt^{D27}$ mutants, it must be both truncated and expressed at very low levels. Nor is the viability of the $In(2)syt^{D27}$ larvae likely to come from a store of synaptotagmin protein or transcript deposited in the eggs by their heterozygous mothers; we have seen no trace of maternally contributed protein or transcript (data not shown). As a further test for synaptic transmission in the absence of synaptotagmin, we constructed embryos with overlapping deficiencies that unequivocally remove the entire *syt* gene. These are extremely sick embryos; they are lacking at least nine essential genes. Nevertheless, these embryos showed extensive movement of head and body wall.

In any genetic analysis the possibility of redundancy must be considered. It is possible that, despite the disruption of *syt* in the $In(2)syt^{D27}$ mutant, there is a second synaptotagmin homolog in Drosophila that produces enough protein to allow transmitter release. We believe that this is an unlikely explanation for our results, because we have failed to find any indication of this hypothetical second gene. When the *syt* gene was originally found by low stringency screening with a probe to p65-A of D. ommata, 13 hybridizing clones were identified, and all were products of the *syt* gene (DiAntonio et al., submitted). Subsequent attempts at low stringency screening have identified no other synaptotagmin homologs. In addition, an antiserum raised to rat synaptotagmin identifies an antigen in the Drosophila neuropil and at neuromuscular junctions. In the $In(2)syt^{D27}$ mutant no immunoreactivity persists at synapses, and thus there is no indication of a second synaptotagmin gene. Had such a hypothetical gene existed, the antiserum we have used would likely have detected its product. This antiserum recognizes some of the best conserved regions of synaptotagmin, including the first C2 domain. The antiserum recognizes rat and fly synaptotagmin and both the A and B isoforms of D. ommata (B. Wendland, personal communication). Thus, any additional synaptotagmin protein in Drosophila must be sufficiently different from these vertebrate forms and the *syt* product itself as to escape detection by this antiserum. There is one precedent for a distantly related member of the synaptotagmin family; in D. ommata there is a C isoform that is more distantly related to the other vertebrate forms than they are to the Drosophila gene. The significance of this form is unclear, since it has not been shown to be a synaptic vesicle protein. A low stringency screen of a cDNA library with this C isoform failed to find a Drosophila homolog. In summary, any additional, redundant gene in Drosophila must be sufficiently different from *syt* on both the nucleic acid level and protein level to escape detection by library screens and antibody staining.

Furthermore, we note that in vertebrates, where multiple synaptotagmin genes have been found, the multiple forms are not redundant with one another but rather are expressed in different cells of the nervous system (Wendland et al., 1991; Geppert et al., 1991). In Drosophila, the single *syt* gene is expressed widely in the nervous system and throughout development. The antiserum that only recognizes the syt protein stains the entire Drosophila neuropil in both embryos and adults and stains neuromuscular junctions in first, second, and third instar larvae. At the very

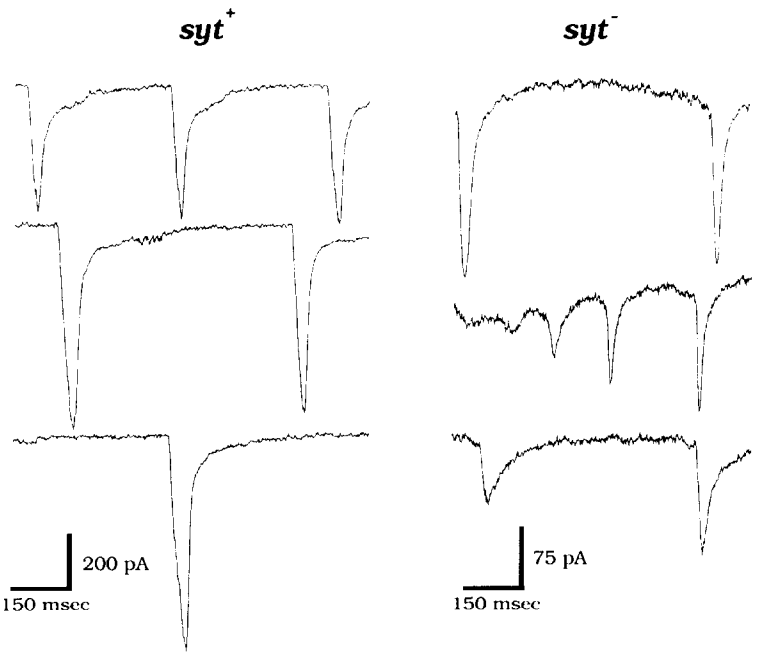

Figure 6. Excitatory Junctional Currents Recorded from Embryos Lacking Synaptotagmin Protein

Spontaneous excitatory junctional currents recorded in whole-cell voltage clamp were observed at neuromuscular junctions of embryos lacking synaptotagmin protein (*syt*$^-$). Embryos were collected from parents carrying the *In(2) syt*^{D27} chromosome over a *CyO* balancer chromosome marked with the β-galactosidase gene. Following physiological recordings, embryos were stained for β-galactosidase activity. Embryos that stained for β-galactosidase were designated *syt*$^+$, and those that did not stain were designated *syt*$^-$. Traces from this *syt*$^+$ were taken 19 hr after egg laying; traces from this *syt*$^-$ were taken 20 hr after egg laying. Both cells were held at −60 mV. Although events from these particular cells differ in amplitude, this difference is not representative of the entire population of *syt*$^+$ and *syt*$^-$ cells. Similar events were observed in three *syt*$^-$ cultures and nine *syt*$^-$ cultures.

synapse most central to our analysis, the neuromuscular junction of the first instar larvae, the *syt* null allele analyzed above removes all detectable syt immunoreactivity.

The neuromuscular junction is currently the clearest example of a synapse in which synaptic transmission occurs in the absence of the *syt* gene product. These neuromuscular junctions use the transmitter glutamate, which is released from small clear vesicles (Jan and Jan, 1976; Johansen et al., 1989; Broadie and Bate, 1993). The rhythmic movements of the late-stage embryos, which we have observed to persist in the mutants, are dependent on neurotransmission (they are abolished by blockers of glutamate receptors; Broadie and Bate, 1993). Likewise, the motor behavior of the larvae clearly indicates that synaptic transmission onto body wall muscles persists in the mutants. Our whole-cell patch clamp recordings at a body wall muscle of embryos have shown that excitatory junctional currents still occur in the *syt* null mutant. Thus, glutamate release from small clear vesicles occurs in the absence of synaptotagmin.

Our data support and extend the results of Shoji-Kasai et al. (1992), who found that a mutant PC12 cell line lacking synaptotagmin was still capable of Ca^{2+}-regulated transmitter release. Release of catecholamines from chromaffin granules was detected with a biochemical assay that measured release on the time scale of minutes. Previous reports have suggested that synaptotagmin may be the fusion protein for synaptic vesicles (Perin et al., 1990) or the Ca^{2+} sensor that triggers fusion (Brose et al., 1992). An essential role for synaptotagmin at either of these steps is not consistent with our data or those of Shoji-Kasai et al., since in either case the lack of synaptotagmin would be expected to block release completely. Our data also do not support an essential role for synaptotagmin in vesicle docking (Petrenko et al., 1991; Bennett et al., 1992); either vesicles can dock in the absence of synaptotagmin, or docking is not a required antecedent to fusion.

The genetic analysis in Drosophila, unlike the work with the PC12 cell line, does indicate that synaptotagmin has an important function in the nervous system. The gross behavioral abnormalities and eventual lethality of the *syt* mutants demonstrate that *syt* is an essential gene in Drosophila. The ability of these defects to be alleviated by a minigene in which *syt* expression is driven by the neuron-specific *elav* promoter demonstrates that the essential function is indeed within the nervous system. One possibility is that two distinct pathways exist for exocytosis in nerve terminals, one that requires synaptotagmin and another that does not. We favor, however, an alternative hypothesis in which synaptotagmin plays an ancillary, modulatory role in regulating the release of vesicles, without being required for the process. Such a role would be consistent with the quantitative changes in exocytosis from PC12 cells that were observed by Elferink et al. (1993). As a modulator of release, the protein may bind Ca^{2+}, or it may facilitate docking or fusion without being essential for those events. In this hypothesis, the sluggishness of the mutant larvae and the failure of the larvae to develop past the first instar stage might result from a quantitative perturbation of transmission. The eventual death of these larvae could, for example, come from malnutrition due to poorly coordinated feeding behavior or from the inappropriate regulation of the hormonal control of molting.

Drosophila will provide an excellent system in which to look for physiological abnormalities due to the absence of synaptotagmin. By recording from embryonic cultures, it should be possible to find the physiological defects that underlie the behavioral deficits of mutant organisms. In addition, we have a number of hypomorphic alleles that survive until the third larval instar, a favorable situation

for a detailed physiological analysis of transmitter release. Moreover, the mutants described here will provide a null background into which altered forms of synaptotagmin can be inserted to elucidate the structural requirements of the protein. Finally, these mutants may also serve as the starting point for the genetic identification of additional genes that function in neurotransmitter release.

Experimental Procedures

Genetics

Fly culture and crosses were performed according to standard procedures. The mutant screen for lethals under *Df(2L)C144* was performed as follows: Male *cn, bw* flies that were isogenic for the second chromosome were fed 25 mM ethylmethane sulfonate (Sigma) as described (Ashburner, 1989) and mated to virgin *BIL²/CyO* females. Single F1 male progeny were pair mated to two virgin *Df(2L)C144/Gla* females. F2 *cn, bw/Gla* male siblings from crosses that failed to produce *cn, bw/Df(2L)C144* flies (phenotypically wild-type wings and eyes) were outcrossed to *Sco/CyO* virgin females. Balanced lines were established that carried individual mutagenized second chromosomes over *CyO*. For the few lines that were lethal over *CyO*, *cn, bw/Sco* males were crossed to *Gla/SM6B* virgin females, and balanced lines were established over *Gla*. Candidate lines were recrossed to *Df(2L)C144/Gla* to confirm the lethality. From 8000 pair matings, 85 lethal mutations were identified. These were deficiency mapped by crossing to *Df(2L)DTD2/Gla* and *Df(2L)Hin34/CyO*. All mutants that were lethal in combination with *Df(2L)C144* and *Df(2L)DTD2* but not *Df(2L)Hin34* were placed into complementation groups by crossing each mutant to every other mutant. If no *cn, bw* flies emerged from a cross that produced at least 100 progeny, then the 2 mutants were considered to be in the same complementation group.

Genomic Southern Blotting

For quantitative Southern blots, each lane was loaded with digested genomic DNA from four adult flies. Blots were probed with a polymerase chain reaction–derived synaptotagmin open reading frame probe and were standardized by hybridization to a *drab3* (Johnston et al., 1991) open reading frame probe. The long and short open reading frame probes were generated by polymerase chain reaction. All Southern blotting was done using ^{32}P-labeled DNA probes. These probes were prepared by standard random priming procedures using [α-^{32}P] dCTP as the labeling nucleotide.

In Situ Hybridization to Drosophila Polytene Chromosomes

The salivary glands of third instar larvae were dissected out in Drosophila Ringer's solution, squashed onto slides, and fixed (Ashburner, 1989). The chromosomes were hybridized with a biotin-labeled DNA probe made to a 30 kb *syt* genomic clone in a 50% formamide buffer. Biotin-labeled probes were synthesized by nick translation, using Bio-16-dUTP (Enzo Diagnostics) as the labeled nucleotide. After washing at 50°C in 2× SSC, the slides were treated with a horseradish peroxidase–avidin complex (DeTek kit, Enzo Diagnostics), and the signal was developed using diaminobenzadine as a substrate for horseradish peroxidase. The chromosomes were counterstained with Giemsa at 1:20 in 0.1 M sodium phosphate (pH 6.9) and mounted in Permount (Fisher).

Assessment of Lethal Period

syt/CyO virgin females were mated to *OrR* (+/+) wild-type males and *syt/+* males, and virgin female progeny were collected. *syt/+* flies were mated to *In(2)syt^{027}/+* flies, and embryos were collected from a 1 hr laying. Embryos were placed in a grid on a clean grape juice–agar plate and allowed to develop at 24°C. The number of larvae that hatched was recorded at 24, 36, and 48 hr after egg laying. For each mutant between 200 and 400 embryos were scored. As a control, *syt/+* flies were mated to +/+ and *DTD52/DTD52* flies, and the same procedure was followed. No difference was observed between +/+ and *DTD52/DTD52*. *DTD52* is the parent chromosome of *In(2)syt^{027}*.

Immunocytochemistry

Whole-mount first instar larval staining was a modification of the third instar procedure of Johansen et al. (1989). Larvae were dissected with microelectrodes and pinned to Sylgard-coated dishes with fine wire. The synaptotagmin primary antibody was provided by K. Miller and B. Wendland and is an affinity purified rabbit polyclonal raised to the cytoplasmic domain of rat synaptotagmin. It was used at a dilution of 1:5000. The signal was developed using the ABC kit (Vector) according to the manufacturer's protocol. The secondary antibody was horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (Vector). Frozen sections of embryos were cut 10 μm thick and adhered to glass slides. Sections were fixed and stained according to a published protocol (Schwarz et al., 1990). The synaptotagmin antibody was used as described above. An anti-β-galactosidase monoclonal antibody (Boehringer Mannheim) was used at a dilution of 1:1300. The secondary antibody was alkaline phosphatase–conjugated horse anti-mouse (Jackson Immunoresearch). Signals were developed with X-phosphate and nitroblue tetrazolium (Boehringer Mannheim) according to the manufacturer's protocol. When double staining embryos, the alkaline phosphatase reaction preceded the peroxidase staining. Sections were mounted in 80% glycerol.

Embryonic Culture

Embryo cultures were made as described by Broadie and Bate (1993). Embryos were dissected and adhered to coverslips approximately 13 hr after egg laying, and the cultures were left in a humid chamber at 24°C. Embryos were cultured in Schneider's media supplemented with insulin, L-glutamine, bicarbonate, penicillin, and 2% fetal calf serum (not heat inactivated) and used at pH 6.9. The mutant stock is balanced over a *CyO* chromosome that is marked with a β-galactosidase gene driven by the nervous system–specific *elav* promoter. Therefore, homozygous mutant embryos will not have β-galactosidase activity. To identify the genotype of an individual embryo following physiological recordings, the embryo was stained for β-galactosidase by incubating the coverslip at 37°C in 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 1 mM $MgCl_2$, and 0.2% X-Gal that had been previously dissolved in dimethyl formamide. After 1 hr embryos were viewed under bright field illumination at 100×. Mutants were identified by the absence of staining. No background β-galactosidase activity was observed in control cultures after 24 hr.

Electrophysiology

For physiological experiments, embryos were cultured in approximately 50 μl of supplemented Schneider's media for 5–8 hr. All whole-cell patch clamp recordings were performed in the supplemented Schneider's media, which was equal in osmolarity to the electrode solution (120 mM KCl, 20 mM KOH, 4 mM $MgCl_2$, 5 mM TES (N-tris [hydroxymethyl]methyl-2-aminoethane sulfonic acid), 5 mM EGTA, 0.25 mM $CaCl_2$, 4 mM ATP, 4 mM GTP, and 36 mM sucrose). The pH of the electrode solution was adjusted to 7.15. Embryos were viewed with 10× eyepieces on an inverted microscope (Nikon) with Hoffman optics at 40×, and recordings were made from muscle 6 of the body wall. The ventral nerve cord and motor neurons of this preparation remain intact and yield action potential–derived synaptic events at the neuromuscular junction (Broadie and Bate, 1993). These spontaneous excitatory junctional currents were recorded at room temperature (20°C–22°C) using standard whole-cell techniques (Hamill et al., 1981; Marty and Neher, 1983) with electrodes that were fire polished to a final resistance of 4–5 Mohm. Whole-cell configuration was achieved with slight suction, and cells were voltage clamped at −60 mV. Signals were amplified with an Axopatch-1C (Axon Instruments) patch clamp amplifier, filtered at 5 kHz, digitized at 2 kHz, and collected on line in 500 ms traces every second.

Many of the whole-cell recordings were interrupted by robust muscular contractions of adjacent fibers, making a thorough quantitative study of possible differences in synaptic transmission between syt$^+$ and syt$^-$ embryos quite difficult. In addition, many of the synaptic events in both syt$^+$ and syt$^-$ embryos were interspersed with atypical, slow onset events that require further investigation.

Acknowledgments

We wish to thank Jeff Sekelsky, Bill Gelbart, and Bruce Baker for providing us with Drosophila stocks, Beverly Wendland and Ken Miller for the gift of their affinity purified anti-synaptotagmin antisera, Felix Schweizer for the data acquisition software, and Kendal Broadie for advice on embryo cultures and recordings. We also wish to thank Irene Inman for excellent technical assistance and Robert Burgess, David Deitcher, and Noreen Reist for discussions and for comments on this manuscript. This work was supported by the McKnight Foundation and a Silvio Conti Center for Neuroscience Award from the National Institute of Mental Health (NIMH). A. D. was supported by a Medical Scientist Training Program fellowship from the National Institute of General Medical Sciences (5T32 GM07365). K. D. P. was supported by a National Research Service Award from the NIMH.

Received February 26, 1993; revised April 16, 1993.

References

Ashburner, M. (1989). *Drosophila*: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Bennett, M. K., Calakos, N., and Scheller, R. H. (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science *257*, 255–259.

Bourne, H. R., Sanders, D. A., and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. Nature *348*, 125–132.

Broadie, K. S., and Bate, M. (1993). Development of the embryonic neuromuscular synapse of *Drosophila melanogaster*. J. Neurosci. *13*, 144–166.

Brose, N., Petrenko, A. G., Südhof, T. C., and Jahn, R. (1992). Synaptotagmin: a calcium sensor on the synaptic vesicle surface. Science *256*, 1021–1025.

Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991). A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. Cell *65*, 1043–1051.

Couteaux, R., and Pecot-Dechavassine, M. (1973). Données ultrastructurales et cytochimiques sur le mechanisme de liberation de l'acetylcholine dans la transmission synaptique. Arch. Ital. Biol. *3*, 231–262.

Elferink, L. A., Peterson, M. R., and Scheller, R. H. (1993). A role for synaptotagmin (p65) in regulated exocytosis. Cell *72*, 153–159.

Geppert, M., Archer, B. T., III, and Südhof, T. C. (1991). Synaptotagmin II. J. Biol. Chem. *266*, 13548–13552.

Gerst, J. E., Rodgers, L., Riggs, M., and Wigler, M. (1992). SNC1, a yeast homolog of the synaptic vesicle-associated membrane protein/synaptobrevin gene family: genetic interactions with the RAS and CAP genes. Proc. Natl. Acad. Sci. USA *89*, 4338–4342.

Hamill, O., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. (1981). Improved patch-clamp techniques for high resolution current recordings from cells and cell-free membrane patches. Pflügers Arch. *391*, 85–100.

Hardwick, K. G., and Pelham, H. R. B. (1992). SED5 encodes a 39-kd integral membrane protein required for vesicular transport between the ER and Golgi complex. J. Cell Biol. *119*, 513–521.

Jan, L. Y., and Jan, Y. N. (1976). Properties of the larval neuromuscular junction in *Drosophila melanogaster*. J. Physiol. *262*, 189–214.

Jessell, T. M., and Kandel, E. R. (1993). Synaptic transmission: a bidirectional and self-modifiable form of cell–cell communication. Cell *72*/Neuron *10* (Suppl.), 1–30.

Johansen, J., Halpern, M. E., Johansen, K. M., and Keshishian, H. (1989). Stereotypic morphology of glutamatergic synapses on identified muscle cells of *Drosophila* larvae. J. Neurosci. *9*, 710–725.

Johnston, P. A., Archer, B. T., III, Robinson, K., Mignery, G. A., Jahn, R., and Südhof, T. C. (1991). rab3A attachment to the synaptic vesicle membrane mediated by a conserved polyisoprenylated carboxy-terminal sequence. Neuron *7*, 101–109.

Leveque, C., Hoshino, T., David, P., Shoji-Kasai, Y., Leys, K., Omori, A., Lang, B., Far, O. E., Sato, K., Martin-Moutot, N., Newsom-Davis, J., Takahashi, M., and Seagar, M. (1992). The synaptic vesicle protein synaptotagmin associates with calcium channels and is a putative Lambert-Eaton myasthenic syndrome antigen. Proc. Natl. Acad. Sci. USA *89*, 3625–3629.

Marty, A., and Neher, E. (1983). Tight-seal whole-cell recording. In Single Channel Recording, B. Sakmann and E. Neher, eds. (New York: Plenum Press), pp. 107–121.

Mochly-Rosen, D., Miller, K. G., Scheller, R. H., Khaner, H., Lopez, J., and Smith, B. L. (1992). p65 fragments, homologous to the C2 region of protein kinase C, bind to the intracellular receptors for protein kinase C. Biochemistry *31*, 8120–8124.

Nonet, M. L., Grundahl, K., Meyer, B. J., and Rand, J. B. (1993). Synaptic function is impaired but not eliminated in C. elegans mutants lacking synaptotagmin. Cell *73*, this issue.

Perin, M. S., Fried, V. A., Mignery, G. A., Jahn, R., and Südhof, T. C. (1990). Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. Nature *345*, 260–263.

Perin, M. S., Johnston, P. A., Ozcelik, T., Jahn, R., Franke, U., and Südhof, T. C. (1991a). Structural and functional conservation of synaptotagmin (p65) in *Drosophila* and humans. J. Biol. Chem. *266*, 615–622.

Perin, M. S., Brose, N., Jahn, R., and Südhof, T. C. (1991b). Domain structure of synaptotagmin (p65). J. Biol. Chem. *266*, 623–629.

Petrenko, A. G., Perin, M. S., Davletov, B. A., Ushkaryov, Y. A., Geppert, M., and Südhof, T. C. (1991). Binding of synaptotagmin to the α-latrotoxin receptor implicates both in synaptic vesicle exocytosis. Nature *353*, 65–68.

Robinow, S., and White, K. (1988). The locus elav of *Drosophila melanogaster* is expressed in neurons at all developmental stages. Dev. Biol. *126*, 294–303.

Schwarz, T. L., Papazian, D. M., Caretto, R. C., Jan, Y.-N., and Jan, L. Y. (1990). Immunological characterization of K^+ channel components from the *Shaker* locus and differential distribution of splicing variants in Drosophila. Neuron *4*, 119–127.

Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M., and Takai, Y. (1993). Rabphilin-3A, a putative target protein for *smg* p25A/*rab*3A p25 small GTP-binding protein related to synaptotagmin. Mol. Cell. Biol. *13*, 2061–2068.

Salminen, A., and Novick, P. J. (1987). A *ras*-like protein is required for a post-Golgi event in yeast secretion. Cell *49*, 527–538.

Segev, N., Mulholland, A., and Botstein, D. (1988). The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. Cell *52*, 915–924.

Shoji-Kasai, Y., Yoshida, A., Sato, K., Hoshino, T., Ogura, A., Kondo, S., Fujimoto, Y., Kuwahara, R., Kato, R., and Takahashi, M. (1992). Neurotransmitter release from synaptotagmin-deficient clonal variants of PC12 cells. Science *256*, 1820–1823.

Sorsa, V. (1988). Chromosome Maps of *Drosophila* (Boca Raton, Florida: CRC Press).

Vogel, U. S., Dixon, R. A. F., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S., and Gibbs, J. B. (1988) Cloning of bovine GAP and its interaction with oncogenic ras p21. Nature *335*, 90–93.

Wendland, B., Miller, K. G., Schilling, J., and Scheller, R. H. (1991). Differential expression of the p65 gene family. Neuron *6*, 993–1007.

Note Added in Proof

The paper referred to throughout as DiAntonio et al., submitted, is now in press: DiAntonio, A., Burgess, R. W., Chin, A. C., Deitcher, E. L., Scheller, R. H., and Schwarz, T. L. (1993). Identification and characterization of *Drosophila* genes for synaptic vesicle proteins. J. Neurosci., in press.