Synaptotagmin controls and modulates synaptic-vesicle fusion in a Ca^{2+}-dependent manner

J. Troy Littleton and Hugo J. Bellen

Although numerous electrophysiological and biochemical studies have defined many of the properties of the putative Ca^{2+} receptor for exocytosis at the synapse, the molecular mechanisms that couple influx of Ca^{2+} and release of neurotransmitter have remained elusive. Several proteins have emerged recently as putative Ca^{2+} sensors. Interestingly, one of these proteins, synaptotagmin, shares many properties with the putative Ca^{2+} receptor. Recent genetic experiments in *Caenorhabditis elegans*, *Drosophila* and mouse have provided important insights about synaptotagmin's role in neurotransmitter release. These experiments, combined with electrophysiological and biochemical studies, suggest that synaptotagmin is a key Ca^{2+} sensor, converting the ubiquitously used cellular secretory pathway into a Ca^{2+}-regulated exocytotic pathway.

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CHEMICAL SYNAPTIC transmission depends upon Ca^{2+}-triggered exocytosis of neurotransmitters from presynaptic terminals. Neurotransmitters are sequestered actively into vesicular organelles known as synaptic vesicles. These vesicles are required for rapid and uniform release of quantal packets of neurotransmitters upon stimulation. The number of quanta released per impulse depends crucially upon the amount of influx of Ca^{2+} during depolarization of the presynaptic terminal. Numerous studies have defined many of the properties of the Ca^{2+} sensor that mediate the activation and fusion of synaptic vesicles with the presynaptic membrane. Recent evidence suggests that the synaptic-vesicle protein synaptotagmin (p65) plays a key role in the Ca^{2+}-sensitive activation of synaptic-vesicle fusion.

An evolutionarily conserved fusion machinery

Many studies, including the identification and characterization of yeast secretory mutants (for review, see Ref. 1), the biochemical characterization of the action of clostridial neurotoxins (for review, see Ref. 2), the molecular interactions between vertebrate synaptic proteins (for review, see Ref. 3), and genetic and electrophysiological studies in vertebrates and invertebrates (for review, see Ref. 4) suggest that a multimeric protein complex is required for synaptic-vesicle docking and fusion. Most components within this complex function ubiquitously in vesicle trafficking, and are conserved from yeast to humans[1]. These conserved trafficking proteins are shown in Fig. 1A, and include the vesicle proteins synaptobrevin and Rab3a, the cytosolic proteins NSF (*N*-ethylmaleimide sensitive fusion protein), SNAP α, β and γ (soluble NSF attachment proteins) and rop (also called nSec1, munc-18 and UNC-18) and the presynaptic membrane proteins SNAP-25 and syntaxin. The current model for the function of these proteins in vesicle trafficking is known as the SNARE (SNAP receptors) hypothesis for exocytosis[5]. The synaptic-vesicle-membrane-specific protein synaptobrevin (v-SNARE) has been shown to interact with the presynaptic membrane proteins SNAP-25 and syntaxin (t-SNAREs), possibly serving to dock synaptic vesicles at the presynaptic membrane or to form an activated, fusion-ready vesicle[5,6]. This protein complex is recognized by the cytosolic SNAP proteins, with subsequent attachment of the ATPase NSF. This larger protein complex is thought to function late in the synaptic-vesicle trafficking pathway, possibly to mediate the final stages of vesicle fusion. Proteins, such as the small GTP-binding protein Rab3a and the syntaxin-binding protein rop, probably participate in the assembly or activation of these protein complexes[7,8]. Given that these proteins function ubiquitously in both the Ca^{2+}-independent constitutive pathway and the Ca^{2+}-regulated fusion pathway, it is unlikely that they mediate the effect of Ca^{2+} on synaptic transmission. Instead, most interest has been directed towards the neuronal specific synaptic-vesicle protein, synaptotagmin.

Biochemical and molecular properties of synaptotagmin

Synaptotagmin was identified originally as an antigen for a synapse-specific antibody[9]. Subsequent studies have demonstrated that synaptotagmin has many properties that suggest that it might play a role in the Ca^{2+}-activated fusion of synaptic vesicles with the presynaptic membrane. The cDNA sequence of synaptotagmin[10] predicts a protein that spans the vesicle membrane once, and has a short amino-terminus intravesicular domain and a larger cytoplasmic carboxy-terminus region (see Fig. 1). Following the transmembrane-spanning region, there are two cytoplasmic repeats that have homology to the regulatory domain (C2 domain) of protein kinase C

J. Troy Littleton and Hugo J. Bellen are at the Howard Hughes Medical Institute, Dept of Molecular and Human Genetics, Division of Neuroscience, Baylor College of Medicine, Houston, TX 77030, USA.

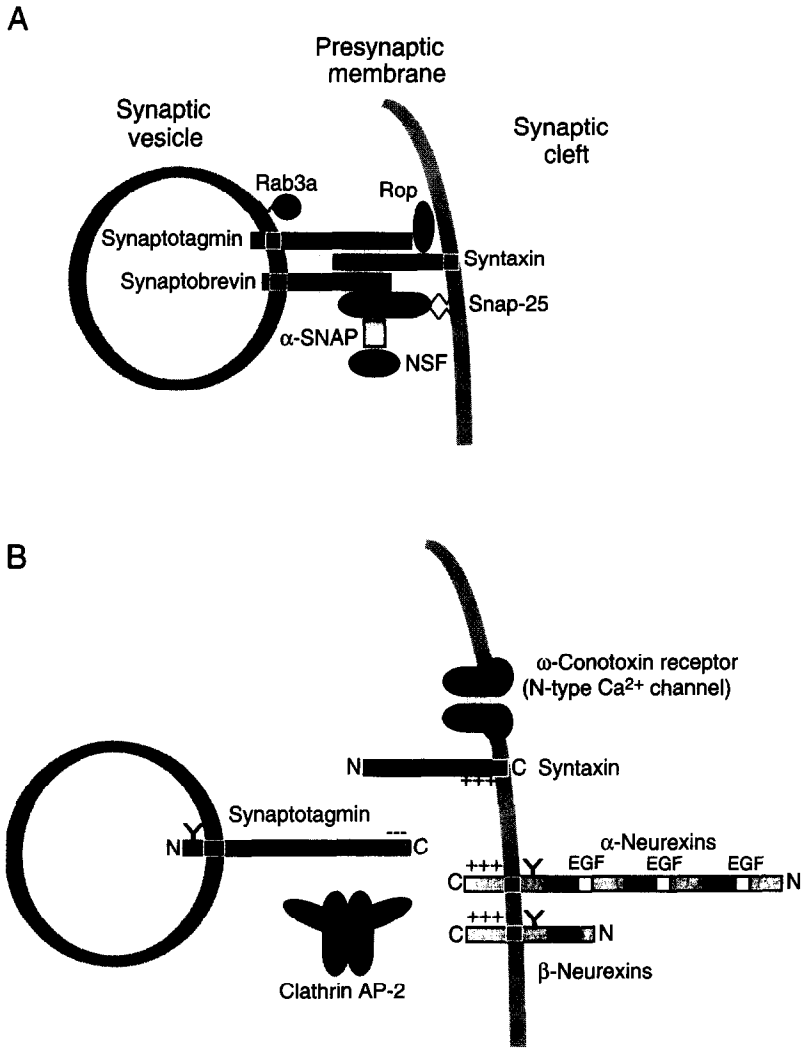

Fig. 1. ***Synaptic fusion machinery and synaptotagmin.*** **(A)** *Synaptic fusion machinery that is thought to function in release of neurotransmitter. For the proposed function of these proteins, see text. All of these proteins, excluding synaptotagmin, have yeast homologues that have been demonstrated to function in vesicular-trafficking pathways.* **(B)** *Domain structure of synaptotagmin and proteins to which it binds. Synaptotagmin contains two cytoplasmic Ca^{2+}-binding C2 domains, and a short highly charged carboxy-terminus tail. This region has been shown to interact with the cytosolic domain of the neurexins, a large family of synaptic extracellular proteins. Synaptotagmin has also been shown to indirectly bind to N-type Ca^{2+} channels through its interaction with the presynaptic-membrane protein syntaxin. Interestingly, there is a small region of homology between syntaxin (KYQSKARRKK) and neurexins (KTPSKAKKNK) close to the inner surface of the presynaptic membrane that is positively charged, and might interact with negatively charged regions of synaptotagmin's carboxy terminus. The second C2 domain of synaptotagmin has also been shown to bind the clathrin-assembly protein AP-2. Not shown is a highly charged potential amphipathic α helix, present in the linker domain between the vesicle membrane and first C2 domain of synaptotagmin, that has been postulated to mediate tetramerization of the protein. Abbreviations: C, carboxy terminus; EGF, extracellular growth factor domain; N, amino terminus; NSF, N-ethylmaleimide-sensitive fusion factor; and SNAP, soluble NSF attachment protein.*

(Ref. 11). This domain has been implicated in Ca^{2+}-associated membrane interactions[10]. Recently, several other proteins[12–14], including rabphilin[15], have also been found to contain a C2-type domain. In addition to two C2 domains, synaptotagmin also contains a charged region that connects the transmembrane moiety with the C2 repeats, and a short carboxy-terminus tail. Synaptotagmin homologues have been identified in humans[16], rodents (four genes identified to date)[10,17–19], fruitflies[16], marine rays[20], nematodes[21] and squid[22]. Sequence comparisons show a striking evolutionary conservation of the two C2 domains and the carboxy-terminus tail[23]. This sequence conservation suggests that these domains are likely to be the most functionally important parts of the protein.

Biochemical and molecular evidence suggests that synaptotagmin might participate in multiple synaptic functions, including fusion, docking, endocytosis, and Ca^{2+} sensing. Evidence that implicates synaptotagmin in vesicle fusion was first reported when bacterially expressed recombinant synaptotagmin protein was shown to bind acidic phospholipids[10]. As synaptotagmin migrates as a tetramer on sucrose gradients, the complex might provide eight potential sites for membrane binding[24]. Additionally, recombinant synaptotagmin, containing the two C2 repeats, is able to bridge membranes as evidenced by its ability to hemagglutinate red blood cells[10]. These *in vitro* data suggest that synaptotagmin is a phospholipid-binding protein that can bridge membranes. Synaptotagmin is localized to most if not all synaptic vesicles[9,20,25], as well as on chromaffin granules[24] and large dense-core vesicles[26]. Hence, synaptotagmin might be a general vesicle-fusion protein, mediating the interaction of several different types of vesicular populations with the presynaptic membrane of neuronal cells. However, direct fusing properties of synaptotagmin have not been observed *in vitro*. In addition, synaptotagmin homologues have not yet been found to be associated with other cellular fusion events, suggesting that synaptotagmin's ability to bind phospholipids might facilitate vesicle fusion that is catalyzed by other proteins.

Synaptotagmin has also been postulated to function as the Ca^{2+} sensor for release of neurotransmitter. Synaptotagmin binds Ca^{2+}, in the presence of phospholipids, in the 10–100 μM range[27]. This range is consistent with the predicted concentrations of Ca^{2+} that are present at the active zone during exocytosis[28]. The first C2 domain of synaptotagmin has been shown to bind Ca^{2+} in a co-operative manner with a Hill coefficient of 2–3 (Ref. 29). Interestingly, co-operative binding of Ca^{2+} had been suggested to be an essential property of the Ca^{2+} sensor[30]. Additionally, anti-synaptotagmin antibodies immunoprecipitate N-type (ω-conotoxin-sensitive) Ca^{2+} channels, suggesting a physical link between the presynaptic Ca^{2+} channel and synaptotagmin[31], and placing the protein at an ideal site to serve as the Ca^{2+} sensor. Thus, synaptotagmin shares many properties with the putative Ca^{2+} receptor for exocytosis, including a similar Ca^{2+} affinity, proximity to the source of influx of Ca^{2+}, and co-operative binding of Ca^{2+}.

Finally, synaptotagmin has also been proposed to function in synaptic-vesicle docking and endocytosis[32]. These roles have been proposed on the basis of its interactions with a number of proteins within the nerve terminal (see Fig. 1B). Synaptotagmin has been shown to interact with syntaxins[33] and presynaptic Ca^{2+} channels[31], possibly providing a mechanism for docking synaptic vesicles at the presynaptic membrane. Synaptotagmin has also been shown to bind to the presynaptic-membrane proteins neurexins[34,35] via a specific interaction between the carboxy-terminus domains of the two proteins[23]. This interaction might have a role in docking or modulation of release of vesicles. Finally, the second C2 domain of synaptotagmin has been

TABLE 1. Analysis of synaptotagmin function

Organism	Manipulations	Observations	Refs
Rat	Synaptotagmin I deficient PC12 cells	Increased release of dopamine and ATP from LDCV	36
	Injection of anti-synaptotagmin antibodies and synaptotagmin protein fragments into PC12 cells	Decreased exposure of dopamine β-hydroxylase resulting from fusion of LDCV	37
Squid	Injection of synaptotagmin peptides into giant synapse	Inhibition of neurotransmitter release, increase in number of docked synaptic vesicles	22
Drosophila	Genetic disruption of *syt* locus, larval recordings from *syt* hypomorphs	Dramatic reduction in elicited release, increased spontaneous vesicle fusion, embryonic lethality, changes in the dependence of neurotransmission on Ca^{2+}	38, 39
	Genetic disruption of *syt*, recordings from rescued transgenic larvae	Behavioral defects, first instar larval lethality, persistence of synaptic transmission, reduced elicited response, increased spontaneous fusion	40, 41
	Embryonic *syt* null recordings	Dramatic decrease in elicited response, increase in spontaneous fusion, majority of nerve stimulations result in failures	42
C. elegans	Genetic disruption of *syt* locus	Behavioral and synaptic defects, accumulation of ACh, co-ordinated muscle movements persist	21
Hamster	Transfection of syt into CHO fibroblasts	Formation of actin-rich filopodial processes after transfection of synaptotagmin	43
Mouse	Genetic disruption of *Syt-I* locus	Decreased fast component of Ca^{2+}-dependent release, no changes in spontaneous vesicle fusion, asynchronous release or number of docked synaptic vesicles	44

Abbreviations: *C.*, *Caenorhabditis*; CHO, Chinese hamster ovary; LDCV, large dense-core vesicles; and *syt*, synaptotagmin gene.

shown to interact with the clathrin-assembly protein AP-2, possibly providing a mechanism for the endocytotic retrieval of synaptic-vesicle proteins from the presynaptic membrane following vesicle fusion[32]. Interestingly, none of these interactions appears to be affected by the concentration of Ca^{2+} *in vitro*.

Functions for synaptotagmin in exocytosis *in vivo*

Given the variety of different roles proposed for synaptotagmin in exocytosis, based on experiments *in vitro*, the role of synaptotagmin in release of neurotransmitter *in vivo* has been a subject of debate. A number of manipulations *in vivo* that involve synaptotagmin have been performed and are summarized in Table 1. Shoji-Kasai and colleagues[36] have shown that uninduced PC12 cells that are deficient for the synaptotagmin I gene can still release dopamine and ATP. However, synaptotagmin III has recently been shown to be expressed abundantly in PC12 cells, possibly compensating for the lack of synaptotagmin I (Ref. 19). Elferink and colleagues[37] have shown that intracellular injection of antibodies against synaptotagmin into PC12 cells induced with nerve growth factor inhibits fusion of large dense-core vesicles. In addition, electrophysiological recordings from the squid giant synapse demonstrate that presynaptic injections of peptides homologous to synaptotagmin's C2 domains block neurotransmission at this synapse[22], suggesting defects in Ca^{2+}-elicited release from small synaptic vesicles also. Collectively, these studies imply that synaptotagmin plays an important role in exocytosis of both small clear synaptic vesicles and large dense-core vesicles.

More direct tests of the role of synaptotagmin in neurotransmission have resulted from genetic disruptions of *synaptotagmin* in *Drosophila*[38–42], *Caenorhabditis elegans*[21] and mouse[44]. These studies have demonstrated continued, but greatly reduced, elicited release in *synaptotagmin* mutants, based both on behavioral and electrophysiological observations (see Fig. 2). These data demonstrate unambiguously a critical role for synaptotagmin in supporting normal levels of synaptic transmission. However, the interpretations from these studies on the precise function of synaptotagmin vary from synaptic-vesicle docking[40,41], endocytosis[21], inhibition of spontaneous fusion[38,39,41,42], and Ca^{2+}-sensitive activation of vesicle fusion[38,39,44]. One caveat to these genetic studies is the possibility of multiple synaptotagmin genes, as observed in rodents[10,17–19] and *C. elegans* (M. Nonet and J. Rand, pers. commun.). These additional isoforms might subserve similar functions and confound the interpretation of synaptotagmin's importance in neurotransmitter release.

To address the function of synaptotagmin in more detail, a closer examination of the precise defects identified in these mutations is required. Upon influx of Ca^{2+} into the presynaptic terminal, fusion of a subset of docked vesicles occurs (for review, see Ref. 45). Reductions of elicited responses could represent a number of perturbations in neuronal function, including failure of synapses to form, reduction in the number of docked synaptic vesicles, loss of the ability of vesicles to fuse, block in endocytosis, or alteration in the Ca^{2+}-mediated activation of vesicle fusion. Several of these functions have been investigated in *synaptotagmin* mutants, and the results lend important insights into precise mechanisms by which loss of synaptotagmin disrupts synaptic transmission.

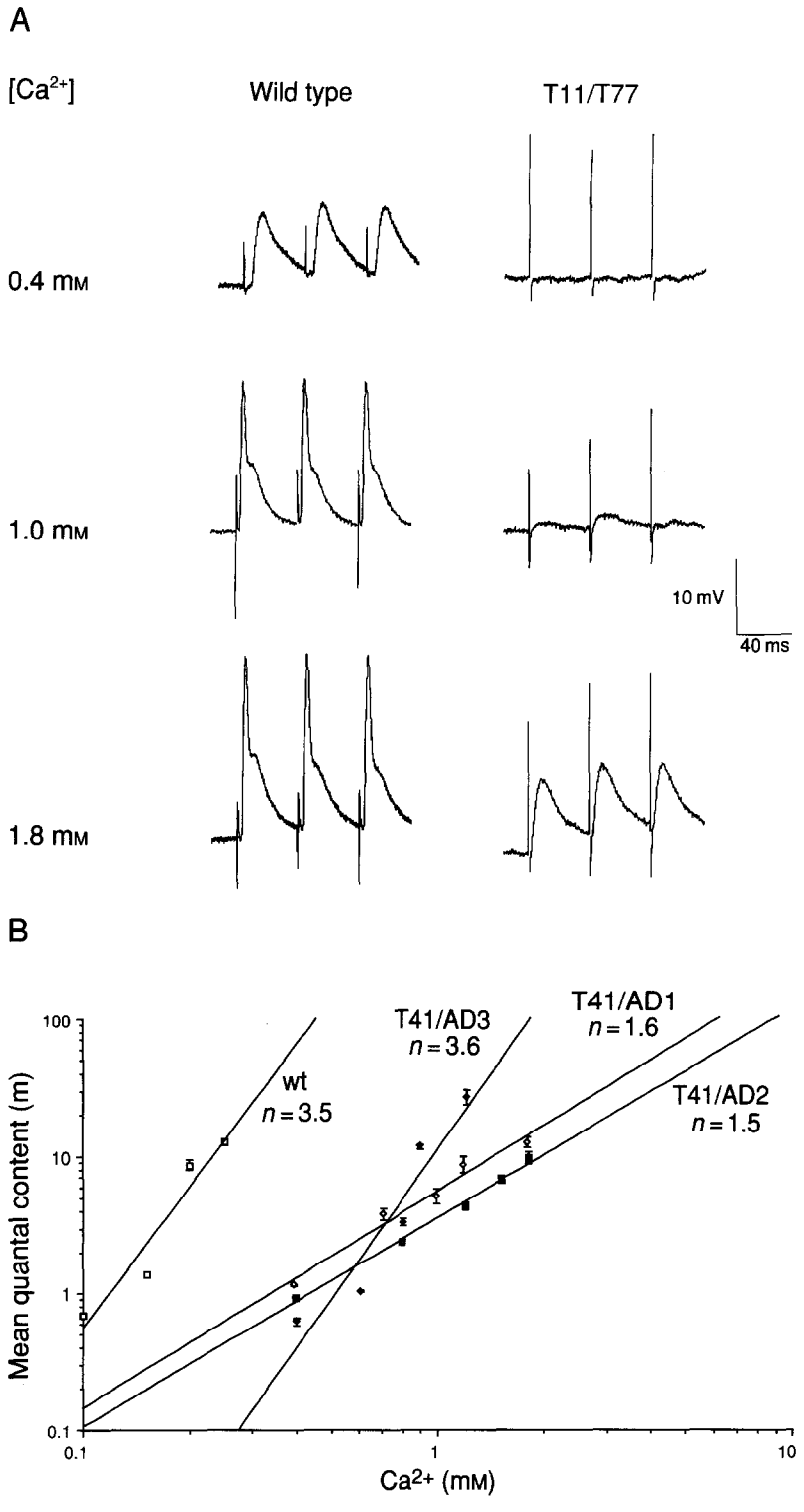

Fig. 2. (Left.) ***Defects in elicited release of neurotransmitter present in* Drosophila *synaptotagmin mutants with partial loss of synaptotagmin function.*** **(A)** *Excitatory junctional potentials were recorded from larval muscle fiber 6 following nerve stimulation at three different concentrations of* Ca^{2+}. **(Left.)** *Wild-type responses.* **(Right.)** *Responses from a* synaptotagmin *mutant (*syt^{T11}/syt^{T77}*) containing reduced levels of the wild-type protein and a mutant allele of* synaptotagmin. *At low extracellular concentrations of* Ca^{2+}*, no responses can be elicited in these mutants. Higher concentrations of* Ca^{2+} *enable partial functioning of the mutant synaptotagmin complex and the elicitation of reduced excitatory junctional potentials.* Reproduced, with permission, from Ref. 38. **(B)** *Dependence of neurotransmitter release of* Ca^{2+} *in* Drosophila synaptotagmin *mutants with partial loss of function of synaptotagmin. A double log plot of the mean quantal content versus the extracellular concentration of* Ca^{2+} *is shown. Wild-type* Drosophila *larvae have slope responses (n) of approximately 3.5. These results have been taken to indicate that three to four* Ca^{2+} *participate in a co-operative manner to elicit a single vesicle fusion*[30]*. One class of* synaptotagmin *mutations shifts this curve to the right, indicating a decrease in quantal content, but does not affect the slope, with neurotransmitter release still dependent upon fourth order* Ca^{2+}*. However, a second class of mutations not only shifts the curve to the right, but also decreases the slope and lowers the dependence of neurotransmitter release on* Ca^{2+} *by approximately half. Thus, depending upon the location of mutations within the synaptotagmin protein, and their interactions as a multimer, an absolute shift in the ability of* Ca^{2+} *to promote release or a change in the order of the dependence of neurotransmitter release on* Ca^{2+} *can result.* Reproduced, with permission, from Ref. 39.

The endocytotic pathway in *synaptotagmin* mutants

A role for synaptotagmin in elongation of axons and formation of synapses is very unlikely, as formation of synapses is unaffected in *synaptotagmin* null mutants[21,38,42]. After the synapse has matured, synaptic vesicles undergo numerous rounds of exocytosis and local recycling. Vesicle recycling is likely to occur via endocytosis by clathrin-coated pits and a cisternal intermediate[46]. Vesicle recycling is not obviously affected in the ten *Drosophila* 'partial loss of function' mutants examined to date[39], including those that lack the second C2 domain. Consistent, though reduced, elicited responses at mutant synapses can be recorded during repetitive stimulation long after depletion of the vesicle pool that would be present at the synapse without endocytosis[38,41]. In addition, mice with reduced levels of a mutated synaptotagmin I protein which lacks the second C2 domain show normal levels of spontaneous vesicle fusions and asynchronous release caused by hypertonic solutions or α-latrotoxin[44], again suggesting that vesicle recycling is replenishing the vesicle populations that are responsible for the observed release. Finally, EM analysis of synaptotagmin-deficient synapses does not show abnormal and enlarged synaptic buttons[22,44], as would be expected for an endocytotic defect. Thus, evidence supporting a role for synaptotagmin in endocytosis *in vivo* is lacking to date. However, it should be noted that the present data do not exclude a role for synaptotagmin in endocytosis, as electrophysiological separation of exocytosis and subsequent endocytosis can be difficult.

Docking in *synaptotagmin* mutants

Following arrival of vesicles at the active zone, docking at presynaptic-release sites must occur. Could the defects that are observed in *synaptotagmin* mutants be a result of defects in docking vesicles at the active zone? Several lines of evidence suggest that the initial phase of vesicle docking is not affected in *synaptotagmin* mutations. First, miniature excitatory junctional potentials (MEJPs), presumably resulting from the fusion of docked synaptic vesicles[47,48], are present at neuromuscular junctions of *synaptotagmin* mutants, and actually occur at increased rates[38,39,41,42]. A second line of evidence arguing against defects in vesicle docking is the consistent amplitude of elicited responses obtained in *Drosophila* mutations with partial loss of synaptotagmin function. Trains of stimuli do not show variable and decreased responses over short periods of time,

as observed when the levels of other proteins that are thought to play a regulatory role in docking such as rop[8] and Rab3a[7] are altered. These observations suggest that recycling and docking occurs at these terminals in a consistent and reproducible pattern[39]. Third, EM studies of terminals with disrupted synaptotagmin function have been performed[22,44]. Bommert and colleagues[22] have shown that peptides derived from the synaptotagmin sequence injected into the giant squid synapse can block neurotransmitter release, but these peptide-treated synapses show an increase in the number of docked vesicles, suggesting defects in activation of vesicle fusion and not docking. In addition, synapses of mutant synaptotagmin I mice also do not show a decrease in the number of docked vesicles at the active zone[44]. However, some evidence is consistent with a role for synaptotagmin in docking. In the complete absence of synaptotagmin, release of vesicles is highly inconsistent following nerve stimulation in *Drosophila* embryos, as 60–70% of stimulations lead to a complete failure of release[42] (see Fig. 3). Such inconsistency in the fusion of vesicles could result from a variable depletion of fusion-ready docked vesicles. Alternatively, these defects can also be explained by a decrease in the probability of release associated with each docked site, or a change in the stability of the docked complex. Hence, the issue of synaptotagmin's function in docking will require further study.

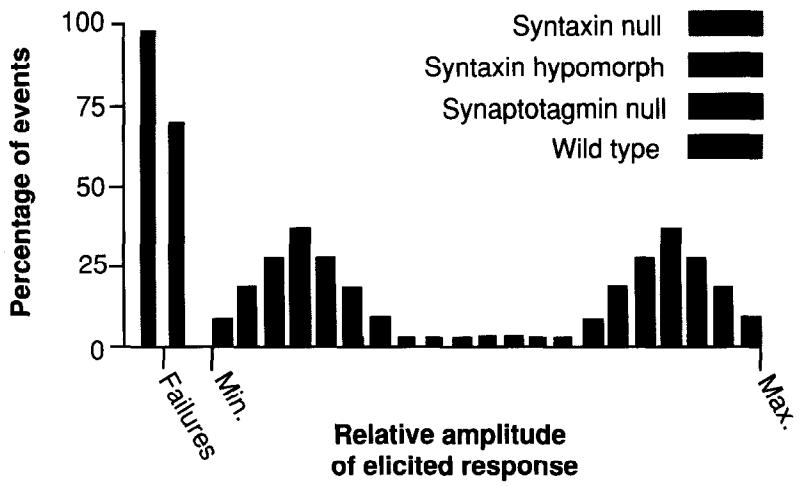

Fig. 3. ***Defects in elicited neurotransmitter release.*** *Comparison of defects in elicited neurotransmitter release at the* Drosophila *embryonic neuromuscular junction of* synaptotagmin *null mutations and* syntaxin *null and hypomorphic mutations in 1.0 mM Ca^{2+}. The percentage of events resulting in a given response versus the relative amplitude compared with the maximum wild-type response is depicted diagramatically. These results are representations of data obtained from Refs 42 and 48, and actual values are not presented, but rather the general shape of the response histogram has been extrapolated. Null mutations in the t-SNARE (SNAP receptor) syntaxin abolishes elicited neurotransmission completely, indicating that the core SNARE complex is essential for synaptic transmission. In contrast, 65–70% of responses result in failures of synaptic transmission in* synaptotagmin *null mutations, with the remaining residual responses scattered throughout the response histogram. This is in sharp contrast to hypomorphic syntaxin mutations, which shift the response histogram to much lower amplitude events, but maintains the typical wild-type bell-shaped response curve. These results indicate that residual synaptic transmission can occur in the absence of synaptotagmin, but that it is unpatterned and variable.*

Spontaneous vesicle fusion is increased in *synaptotagmin* mutants

Following docking at the active zone, vesicles remain in a fusion-ready state until a Ca^{2+} signal is received. Several results suggest that synaptotagmin plays a critical role in the maintenance of synaptic vesicles in the docked state. Evidence supporting this role includes the increase in frequency of MEJPs in *synaptotagmin* mutants[38,39,41,42]. This activity supports a role for synaptotagmin as a vesicle clamp to block release in the absence of Ca^{2+}. This modification to the constitutive pathway by neurons could enable the maintenance of a population of fusion-ready vesicles, providing a necessary component for rapid synaptic transmission. Mice with reduced levels of mutated synaptotagmin I show no increase in the frequency of MEJPs (Ref. 44), but this function might be masked by other synaptotagmins. Interactions with syntaxins[33] or neurexins[35] might enable synaptotagmin to modulate the constitutive pathway and prevent spontaneous fusion in the absence of Ca^{2+}. An additional interaction that might mediate the ability of synaptotagmin to serve as a clamp is the finding that α-SNAP can displace binding of synaptotagmin to the SNARE complex, indicating that these two proteins share common binding sites on the complex[49]. It is likely that additional synaptic proteins, such as rop[6,8], also function to stabilize or modulate docked synaptic vesicles. Further work will be required to determine precisely how spontaneous vesicle fusion increases in the absence of synaptotagmin.

Synaptotagmin is a Ca^{2+} sensor for neurotransmitter release

Multiple lines of evidence implicate synaptotagmin in Ca^{2+} sensing and activation of synaptic-vesicle fusion. First, elicited responses in *synaptotagmin* mutants are decreased dramatically, implying that synaptotagmin function is necessary for the majority of elicited release[38,42,44]. Second, synaptotagmin is absolutely required for vesicle fusion at the *Drosophila* larval neuromuscular junction in low concentrations of Ca^{2+} (Ref. 38). Third, excitation–secretion coupling in *Drosophila synaptotagmin* null mutations shows little sensitivity to external Ca^{2+}, with the majority of elicited responses resulting in failures of transmission[42] (see Figs 3 and 4). Fourth, the fast synchronous component of elicited release is abolished in mice with mutated synaptotagmin I (Ref. 44). Finally, a number of different *Drosophila synaptotagmin* mutations decreases the order of the dependence of neurotransmitter release on Ca^{2+} by half (n) (from $n = 3.5$ to $n = 1.6$) at mature third instar larval synapses, including mutants that contain proteins that lack the second Ca^{2+}-binding domain[39] (see Figs 2 and 4). These results are quite different from the data obtained by recording from immature synapses in *Drosophila* embryos that completely lack synaptotagmin. At this immature synapse, the order of the dependence on Ca^{2+} is not changed. However, at this synapse $n = 1.8$ in both wild-type and *synaptotagmin* null embryos, and is thus very different from the measurements obtained at most mature synapses where $n = 3–4$. These results suggest a developmental change in n accompanies synapse maturation, and that synaptotagmin is probably an essential protein involved in the formation of the functional properties of a mature synapse. These observations are most consistent with synaptotagmin being a Ca^{2+} receptor responsible for activation of synaptic-vesicle fusion. This function

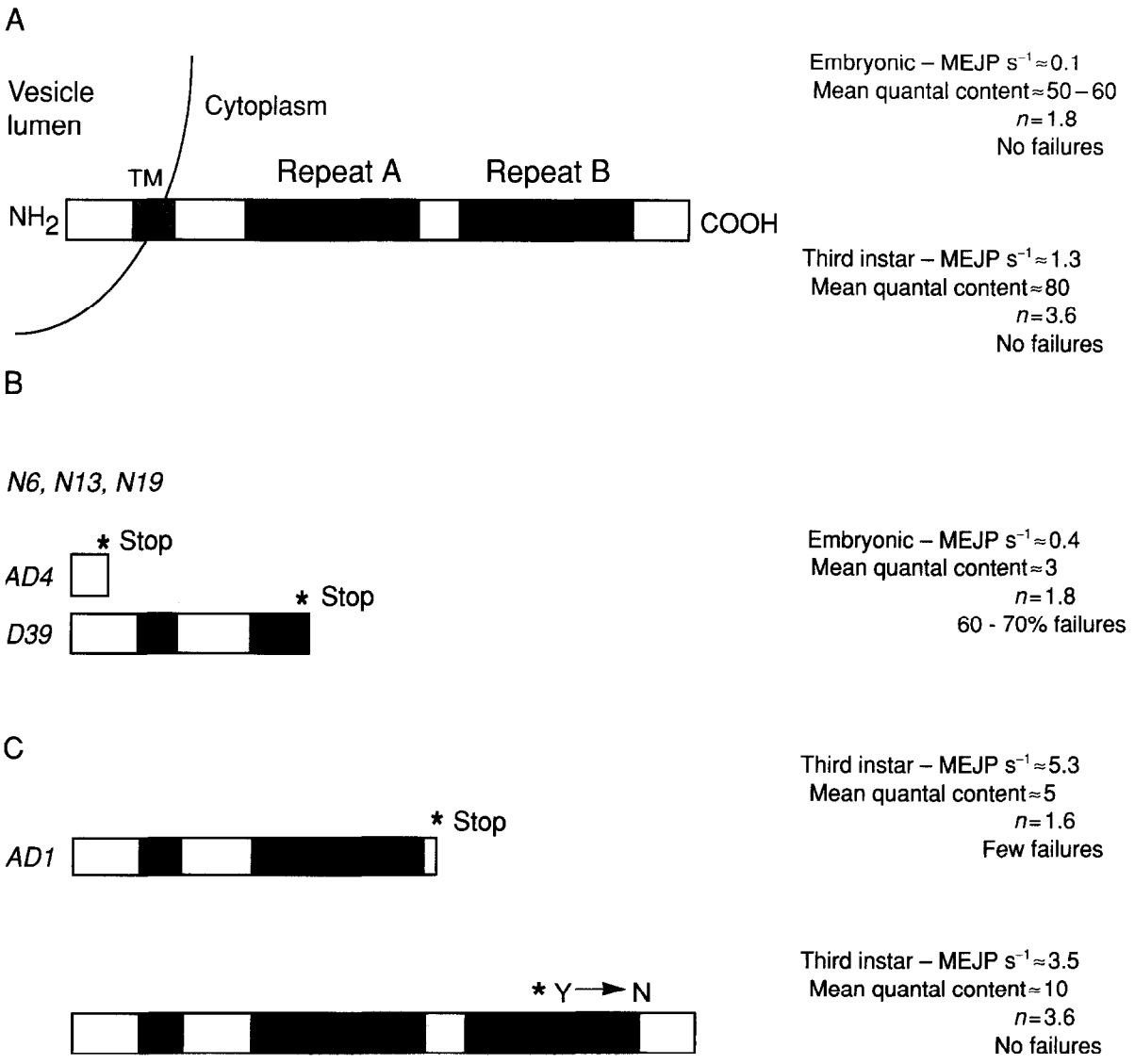

Fig. 4. ***Structure–function analysis of synaptotagmin.*** *A number of the molecular defects that are present in* Drosophila synaptotagmin *mutations have been identified[39,41] and correlated with the electrophysiological defects that are present at muscle fiber 6 in these mutants at approximately 1 mM Ca^{2+} (Refs 39 and 42).* **(A)** *The wild-type domain structure of synaptotagmin is depicted with the corresponding electrophysiological properties of the embryonic and third instar larval synapse. Maturation of the wild-type response from embryos to third instar larva results in an increase in spontaneous fusion (minis) and mean quantal content accompanying the increase in number of boutons that synapse upon muscle fiber 6 during development. In addition, there is a developmental change in the order of dependence of neurotransmission on Ca^{2+} (*n*) from n = 1.8 in the embryo to the more commonly recorded value of n = 3.6 in third instar larva.* **(B)** *Null mutations in* synaptotagmin *have been identified as those lacking at least one complete C2 domain (*N6, N13, N19, AD4, D39*). Thus, to obtain any residual synaptotagmin function in exocytosis at least one complete Ca^{2+}-binding motif must be present. Null alleles of synaptotagmin are embryonic[38] or first instar lethal[40] and result in a ten- to twentyfold decrease in quantal content and a fourfold increase in the rate of spontaneous vesicle fusion. In addition, there are dramatic increases in failure rate. The dependence of neurotransmitter release of the immature embryonic synapse on Ca^{2+} appears unaltered with n = 1.8.* **(C)** *Third instar larvae with residual synaptotagmin function have been obtained through intragenic complementation between partial loss of function mutations[39], suggesting synaptotagmin functions as a multimeric complex. Many of these complexes contain either the alleles* AD1 *or* AD3 *paired with additional mutations.* AD1 *has been shown to lack the second C2 domain, whereas* AD3 *contains a single amino acid change in the second C2 domain[41]. Removing one Ca^{2+}-binding domain from synaptotagmin (*AD1*) causes a decrease in the co-operativity of Ca^{2+} ions required to elicit a vesicle fusion from 3.6 to 1.8. A point mutation in the second Ca^{2+}-binding domain (*AD3*) does not affect the co-operativity of binding of Ca^{2+}, but instead decreases the affinity for Ca^{2+} in promoting vesicle fusion. Mutant complexes containing* AD1 *or* AD3 *also result in an increase in spontaneous vesicle fusion.*

of synaptotagmin as a Ca^{2+}-sensitive activator of vesicle fusion is clearly distinct from its role as a clamp that prevents spontaneous fusion. The activation of vesicle fusion can be separated electrophysiologically from the increase in the rate of spontaneous vesicle fusion[39,41,44], indicating that activation of vesicle fusion is not simply a function of synaptotagmin releasing a clamp on the constitutive fusion pathway. Given the persistence of spontaneous vesicle fusions and asynchronous release in synaptotagmin mutations, it is unlikely that synaptotagmin would participate in the actual fusion reaction. Rather, the phospholipid-binding activities of synaptotagmin might enable the protein to pull the vesicle into closer contact with the presynaptic membrane, increasing the probability of vesicle fusion mediated by the constitutive SNARE fusion machinery. Alternatively, Ca^{2+}-induced conformational changes in synaptotagmin might be relayed to proteins, such as syntaxins or neurexins, through which vesicle fusion is initiated.

Concluding remarks

The residual Ca^{2+}-dependent release in *synaptotagmin* null alleles[21,25,40,42] and in mice containing mutated synaptotagmin I (Ref. 44) indicates that there must be additional proteins that also function in mediating the effect of Ca^{2+} on exocytosis. The identity of these additional Ca^{2+}-sensing proteins is currently unknown, but potential candidates include the Rab3a-binding protein rabphilin[15] and the synaptic cytosolic protein frequenin[50]. In conclusion, it is likely that synaptotagmin represents a neuronal-specific modification to the constitutive vesicle-fusion pathway, providing a mechanism to both prevent spontaneous fusion in the absence of Ca^{2+} and activate the constitutive fusion pathway during influx of Ca^{2+} by functioning as a Ca^{2+} sensor.

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Glycans and the modulation of neural-recognition molecule function

Melitta Schachner and Rudolf Martini

Neural-recognition molecules are carbohydrate-bearing glycoproteins, glycolipids or proteoglycans that are found at the cell surface or in the extracellular matrix that regulate cell interactions during development, modification of synaptic activity and regeneration of nerve connections after damage in the adult. The expression of the carbohydrates appears to be fine tuned to these functions. Among the identified carbohydrates are polysialic acid, a 3′-sulfated glucuronic acid, and oligomannosidic residues. They act not only between apposing partner cell surfaces (*trans*-interaction) but also between recognition molecules within the surface membrane of one cell (*cis*-interaction), thereby forming complexes that influence transduction of signals to the cell interior.

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THE FINE TUNING of cell interactions is an important requirement for the specification of nerve-cell contacts that ultimately underlie the orderly development of neural networks. Cellular behaviour in the complex environment of the nervous system is shaped by recognition molecules at the cell surface and in the extracellular matrix that regulate cell interactions by forming molecular complexes with appropriate target molecules, most likely in a lock-and-key manner, and by transducing the recognition signal to the cell interior. Signal transduction mechanisms that involve the cascades of second messenger systems within the cell have been reported to impinge on ion channels and the cytoskeleton,

Melitta Schachner and Rudolf Martini are at the Dept of Neurobiology, Swiss Federal Institute of Technology, Hönggerberg, 8093 Zürich, Switzerland.