

Genetic dissection of synaptic transmission in *Drosophila*

Mark N Wu* and Hugo J Bellen†

Recent studies of mutations of *Drosophila* proteins implicated in synaptic transmission have yielded new insights into the roles of these proteins and the pathways in which they function. Analysis of mutant embryos lacking syntaxin or synaptobrevin suggests that these proteins perform distinct functions after vesicle docking with the presynaptic membrane. In addition, characterization of *Drosophila* endocytotic mutants provides *in vivo* evidence for the presence of different endocytotic pathways at a single synapse.

Addresses

*Howard Hughes Medical Institute, Department of Molecular and Human Genetics, Division of Neuroscience, Baylor College of Medicine, Houston, Texas 77030, USA

†Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, USA

†e-mail: hbellen@bcm.tmc.edu

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Abbreviations

AP	adaptor protein
NMJ	neuromuscular junction
NSF	<i>N</i> -ethylmaleimide-sensitive factor
n-syb	neuronal synaptobrevin
SNAP	soluble NSF attachment protein
SNAP-25	synaptosome-associated protein of 25 kDa
SNARE	SNAP-associated receptor
syb	synaptobrevin
syx	syntaxin-1A
TNT	tetanus toxin
t-SNARE	target membrane SNARE
v-SNARE	vesicle SNARE

Introduction

One of the continuing challenges for neurobiologists is the development of a mechanistic understanding of synaptic transmission. Fast chemical synaptic transmission is mediated by neurotransmitter-containing synaptic vesicles that rapidly fuse with the presynaptic membrane in response to an influx of Ca²⁺. These synaptic vesicles are then locally retrieved and recycled for continued use [1–3]. Over the past decade, a combination of biochemical, cell biological, and genetic approaches has joined morphological and electrophysiological studies in attempting to unravel the molecular mechanisms of synaptic vesicle exocytosis and endocytosis.

Drosophila has recently emerged as an excellent model organism for studying the function of the synapse [4–7]. In general, proteins involved in synaptic transmission in *Drosophila* appear well conserved when compared with their vertebrate homologs, suggesting that insights gained

from studies in *Drosophila* are generally applicable to vertebrates. In addition, genetic analyses in *Drosophila* are less hampered by the difficulties arising from the presence of multiple isoforms of a protein, which can cloud the interpretations of mutant phenotypes. However, one of the greatest strengths of *Drosophila* for studying synaptic function lies in the ability to combine sophisticated genetic techniques with detailed electrophysiological analyses in both larval and embryonic stages [8–10]. In particular, the ability to assay electrophysiological phenotypes in mutant embryos allows for the functional analysis of essential genes. Here, we review recent insights gained from genetic studies into the mechanisms underlying exocytosis and endocytosis of synaptic vesicles in *Drosophila*.

Testing the SNARE hypothesis in *Drosophila*

There are at least three key steps in synaptic vesicle exocytosis: the targeting and docking of synaptic vesicles to the active zone; the ‘priming’ of these vesicles; and the fusion of the vesicles with the presynaptic membrane in response to an influx of Ca²⁺ following an action potential [3]. Several years ago, Rothman and colleagues [11] proposed the ‘SNARE’ hypothesis, which suggested that the proper targeting and docking of vesicles is mediated by a cognate interaction between v-SNAREs (vesicle SNAP-associated receptors, where SNAP is soluble *N*-ethylmaleimide-sensitive factor [NSF] attachment protein) and t-SNAREs (target membrane SNAREs). Two synaptic vesicle proteins, synaptobrevin and synaptotagmin, are proposed to act as v-SNAREs, whereas two presynaptic membrane proteins, syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa), correspond to t-SNAREs [11,12]. The fusion of the vesicle with the membrane was proposed to be driven by the action of the homomultimeric ATPase NSF after its recruitment to the SNARE complex by SNAPs. Although these proteins are clearly important in exocytosis, studies in *Drosophila*, neuroendocrine cells, and yeast have challenged the SNARE hypothesis. These studies suggest that NSF does not directly participate in the fusion event itself [13–15,16*,17*] and that v- and t-SNARE proteins perform distinct functions after, not before, the docking of vesicles to the membrane [18–22].

The amount of information gained from the characterization of *Drosophila* mutants that are defective in synaptic vesicle exocytosis is growing rapidly and suggests that each protein implicated in this process performs nonredundant and specific functions in secretion (Table 1) [4,5]. In addition to NSF and the SNARE proteins, other proteins such as ROP (Ras opposite) and cysteine string proteins (CSPs) have been implicated in the regulation of synaptic vesicle exocytosis in *Drosophila* (Table 1). However, because of the current controversy regarding the

SNARE hypothesis, we focus here on recent studies on NSF and SNARE proteins in *Drosophila*.

NSF

There are two NSF genes in *Drosophila* (*comatose*, which encodes dNSF1, and *dNSF2*), and mutations in both of these genes have been isolated ([23–25]; L Pallanck, personal communication). Phenotypic characterization of these mutants is ongoing. Although NSF has been shown to be required for general secretory events [26,27], the strongest *in vivo* evidence for a role for NSF in synaptic transmission has come from the discovery that mutations in dNSF1 (*comatose*) cause a temperature-sensitive paralytic phenotype [25,28,29]. Classic studies performed by Siddiqi and Benzer in 1976 [28] indicated that the kinetics of onset to and recovery from temperature-sensitive paralysis of *comatose* mutants are significantly slower than those of *shibire* flies, which are impaired in dynamin function and which are defective in endocytosis. As clathrin-mediated endocytosis is thought to occur on the order of tens of seconds and fusion of docked synaptic vesicles occurs on the order of hundreds of microseconds, these findings may imply that NSF acts in a slower process, such as the priming of vesicles. In the past year, studies in PC12 cells and yeast have supported a role for NSF in the priming, not the fusion, of vesicles [15,16*,17*].

Syntaxin

Syntaxin-1 is a 35 kDa presynaptic membrane protein, originally identified by virtue of its interaction with synaptotagmin [30]. The SNARE hypothesis suggests that syntaxin is a t-SNARE and participates in the targeting and docking of vesicles. However, syntaxin, as well as SNAP-25, is found along the entire length of the axon, not just at the synapse, making it unlikely that these t-SNAREs are the sole source of targeting specificity [18,31–33]. *syntaxin-1A* (*syx*) mutants have been identified

in *Drosophila*, and phenotypic analysis of these mutants indicates that syntaxin is essential for neuronal as well as non-neuronal secretion [18,31].

syx null mutants reveal a complete absence of both evoked and spontaneous neurotransmitter release at the neuromuscular junction (NMJ). These defects are presynaptic in origin, as the postsynaptic muscle cell can respond normally to glutamate iontophoresis [18]. Although no neurotransmitter release is observed, ultrastructural studies of *syx* null mutants show that vesicles appear normally docked at the active zone and that there is a significant increase in the number of docked vesicles [20]. Further, the docked vesicles in *syx* mutants are mature, functionally docked vesicles, as they can be released by hypertonic saline, which is thought to cause the release of vesicles from the docked, readily releasable pool [34,35]. However, the miniature excitatory junctional currents that are induced by an increase in osmolarity are not normal in *syx* mutants; the amplitudes of these currents are reduced and highly variable compared with controls [20]. This observation suggests that in *syx* mutants, under conditions where some synaptic vesicles can be triggered to fuse with the presynaptic membrane, the absence of syntaxin impairs the ability of these vesicles to undergo full fusion. Alternatively, it is possible that syntaxin controls the amount of neurotransmitter within synaptic vesicles, but there is no evidence for syntaxin performing such a function from any system studied thus far. Therefore, syntaxin is not itself required for the docking of synaptic vesicles with the presynaptic membrane, but appears to be essential for the fusion event.

Synaptobrevin

In *Drosophila*, two synaptobrevins have been identified—a neuronal synaptobrevin (encoded by *n-syb*) and a

Table 1

Drosophila* mutants in synaptic vesicle exocytosis.

Protein	Mutant	Lethality	Evoked response	Spontaneous response	Ultrastructural morphology	Acts in non-neuronal secretion?	Suggested function	References
dNSF1	<i>com</i> (<i>comatose</i>)	Temperature-sensitive paralytic	↓	?	?	No?	Priming of SV?	[25,28]
Synaptotagmin	<i>syt</i>	Embryonic	↓	↑	?	No	Ca ²⁺ sensor; inhibits spontaneous release; required for excitation–secretion fidelity	[65–67]
Syntaxin	<i>syx</i>	Embryonic	Blocked	Blocked	Increase in docked vesicles	Yes	Essential for fusion of SVs	[18,20,31]
n-Synaptobrevin	<i>†</i>	Embryonic	Blocked	↓	Increase in docked vesicles	No	Key function in controlling exocytosis	[19,20]
ROP (n-sec1 homolog)	<i>rop</i>	Embryonic	↓ or ↑	↓	?	Yes	Rate-limiting regulator that performs both positive and negative roles	[68,69] (a)
Cysteine string protein	<i>csp</i>	Variable (adult escapers)	↓	Normal	?	No?	May stabilize an interaction between the SV and Ca ²⁺ channels	[70,71]

*This table lists mutations of *Drosophila* proteins implicated in synaptic vesicle exocytosis, together with electrophysiological, ultrastructural and general characteristics associated with each mutant. The electrophysiological phenotypes are quite specific for each mutant, suggesting that the proteins in this table perform distinct functions in exocytosis. †These results are based on studies of flies overexpressing TNT. (a)MN Wu *et al.*, unpublished data. SV, synaptic vesicle.

ubiquitous synaptobrevin (encoded by *syb*) [36–38]. Mutations in both genes have been isolated ([6]; B McCabe, personal communication), and phenotypic characterization is in progress. An elegant alternative approach to ablating n-syb function was employed by Sweeney *et al.* [19], who generated flies that express tetanus toxin (TNT) exclusively in neurons, thereby enabling cleavage of solely n-syb. In flies that express TNT presynaptically, n-syb is undetectable, and these flies die as mature embryos. Although no defects in muscle or nervous system development are observed, these embryos are paralyzed. Electrophysiological recordings at the NMJs of these mutants show that they completely lack evoked neurotransmission, but 50% of spontaneous neurotransmission persists [19]. This suggests that n-syb is absolutely required for evoked, but not spontaneous, neurotransmission. As with *syx* null mutants, TNT-expressing embryos exhibit an increase in the number of mature, docked vesicles, similar to that observed at TNT-treated squid synapses [21]. However, unlike in *syx* mutants, the miniature excitatory junctional currents in TNT embryos elicited by hypertonic saline resemble those in wild-type controls [20].

The analyses of *syx* mutant and TNT-expressing flies indicate that both syntaxin and n-syb are not themselves essential for the docking of synaptic vesicles and, furthermore, suggest that n-syb function is different from syntaxin function in secretion. The analysis of TNT-expressing embryos is, however, subject to two caveats. First, it is possible that, although undetectable by immunocytochemical means, some n-syb remains at the synapses of TNT embryos that may allow for the observed 50% of spontaneous neurotransmitter release. Second, TNT does not cleave the ubiquitous synaptobrevin, which might also mediate residual spontaneous vesicle fusions [19]. Both of these caveats should be resolved with the study of *syb* and *n-syb* mutants.

However, several lines of evidence from the use of botulinum toxin and yeast studies do support the notion that synaptobrevins are not as critical for exocytosis as t-SNAREs and probably play a distinct role in secretion. First, early studies utilizing clostridial neurotoxins, prior to the knowledge of their molecular targets, grouped the toxins into two groups that differed in their abilities to block spontaneous release. Botulinum toxins B, D, and F (which cleave synaptobrevin) were in some cases ten times less effective than botulinum toxins A and E (which cleave SNAP-25) at reducing spontaneous release [39–42]. Second, in yeast, there are two known synaptobrevin homologs (SNC1 and SNC2) and two syntaxin homologs (SSO1 and SSO2) at the plasma membrane [43,44]. While removal of SSO1 and SSO2 causes unconditional lethality, deletion of SNC1 and SNC2 causes only conditional lethality at elevated temperatures and does not eliminate secretion. Third, in a yeast vacuolar fusion assay, removal of the syntaxin homolog causes more severe defects in all cases than removal of the synaptobrevin homolog

[17*]. Although it remains possible that unidentified synaptobrevin isoforms may partially compensate for the loss of known isoforms, these findings, coupled with the analysis of TNT-expressing flies, strongly suggest that, unlike syntaxin, synaptobrevin is not essential for the fusion of synaptic vesicles, but probably performs a key role in controlling the fusion process.

Spatial and temporal dynamics of different endocytotic pathways

Equally important as exocytosis for synaptic transmission is the recycling of synaptic vesicles through endocytosis. Although it is clear that membrane is rapidly retrieved after exocytosis, there is still controversy over the precise mechanisms underlying the pathways mediating this retrieval [45,46]. Early ultrastructural and biochemical studies suggested that endocytosis of synaptic vesicles is a faster, specialized form of the general clathrin-mediated endocytotic pathway [47–49]. During this process, the AP2 adaptor protein complex is recruited to the membrane and triggers the formation of a clathrin coat, leading to the budding of the vesicle [50,51]. In addition to this clathrin-mediated pathway, it has recently been suggested that, under moderate stimulation conditions, another, faster endocytotic pathway may exist—a kiss-and-run mechanism, whereby vesicles transiently and partially fuse with the membrane to discharge their contents and then quickly separate, never completely fusing with the presynaptic membrane [52–55].

The recent analysis of mutations in α -adaplin (a subunit of the AP2 complex), coupled with recent studies capitalizing on temperature-sensitive mutations in the GTPase dynamin (which is encoded by *shibire*), functionally dissect two stages of clathrin-mediated endocytosis, namely, vesicle budding and vesicle fission [56•,57,58•]. These studies support an essential role for clathrin-coat-mediated endocytosis in synaptic transmission. Furthermore, immunocytochemical and ultrastructural analyses suggest that two distinct endocytotic pathways may exist, each with distinct temporal and spatial dynamics.

α -adaplin

α -adaplin has recently been identified in *Drosophila* [56•]. It is a subunit of the heterotetrameric AP2 complex, which also includes β -adaplin, AP50, and AP17. As mentioned above, the AP2 complex is thought to trigger the assembly of clathrin subunits into a lattice. In addition, the AP2 complex has also been shown to bind the GTPase dynamin *in vitro*, and, hence, may recruit it to the budding vesicle [59]. Dynamin is required for the fission of the vesicle from the membrane (Figure 1), as demonstrated by the presence of ‘collared pits’ at *shibire* mutant synapses and the presence of tubular invaginations coated with dynamin at nerve terminals treated with GTP γ S, which inhibits dynamin activity [60,61]. Characterization of *Drosophila* α -adaplin mutants shows that clathrin-mediated endocytosis is essential for synaptic transmission. First,

utilizing FM1-43, it was observed that endocytosis is impaired in a partial loss-of-function mutant [56**]. FM1-43 is a styryl dye that intercalates into the outer leaflet of membranes and is commonly used as a marker for exocytosis and endocytosis [62]. Further, α -adaptin null mutant synapses lack synaptic vesicles and instead reveal deep folds in the membrane, suggesting that endocytosis was blocked and that the blockage occurs before the formation of clathrin-coated pits [56**]. In contrast, collared pit structures can be observed in *shibire* mutant synapses [60], suggesting that α -adaptin and dynamin act sequentially in endocytosis, participating in vesicle budding and fission, respectively. Finally, α -adaptin mutants demonstrate a range of locomotor defects, ranging from embryos that are unable to hatch (null mutants) to uncoordinated adults that are unable to walk or fly (weak partial loss-of-function mutants) [56**]. These studies demonstrate that α -adaptin is required for vesicle budding and that this function is essential for synaptic transmission.

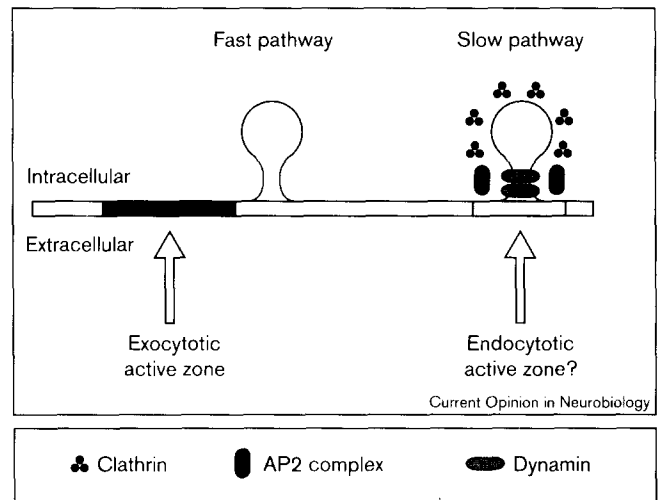
Endocytotic 'active zones'

Classic freeze fracture studies of the frog NMJ showed that, after exocytosis, particles representing synaptic vesicle proteins moved away from the active zone before internalization, suggesting that there may be specific regions for endocytosis [63]. Recently, Estes *et al.* [57] depleted *shibire* synapses of synaptic vesicles and examined the localization of dynamin at the presynaptic membrane. Strikingly, dynamin was localized in a punctate distribution on the membrane, suggesting that it may be associated with presynaptic components that tether it near potential endocytotic active zones. As mentioned earlier, dynamin binds the AP2 complex *in vitro*, and confocal microscopy demonstrates that α -adaptin is also localized in a punctate manner on the presynaptic membrane, similar to dynamin [56**]. The punctate distribution of α -adaptin is not disturbed in *shibire* mutants, suggesting that α -adaptin acts upstream of dynamin, probably by tethering it near the endocytotic active zone. Therefore, clathrin-mediated endocytosis appears to occur at endocytotic active zones.

A fast endocytotic pathway

In addition to clathrin-mediated endocytosis, several lines of evidence have suggested that a faster form of endocytosis may occur [53,54]. Recently, evidence for the presence of two distinct endocytotic pathways in an individual synaptic terminal was obtained by taking advantage of the *shibire* mutation [58*]. At 29°C, all endocytotic activity is blocked in temperature-sensitive *shibire* mutants. However, at 26°C, vesicle recycling begins, but vesicles are unable to pinch off. Ultrastructural analyses of *shibire* synaptic terminals at this temperature reveal the presence of two sites for endocytosis. At 26°C, membrane begins to accumulate as flat cisternae at the exocytotic active zone in less than one minute, and this pathway does not appear to involve intermediate structures. After more than two minutes, collared pits appear away from the exocytotic active zone, at specific

Figure 1



Schematized here are two different endocytotic pathways at a single synapse in *Drosophila*. A fast endocytotic pathway retrieves vesicle membrane near the exocytotic active zone. This pathway does not involve intermediate structures and could potentially correspond to a 'kiss-and-run' mechanism, where vesicle fusion with the presynaptic membrane is transient and partial. A slower, clathrin-mediated pathway occurs at distinct locations away from the exocytotic active zone, and these specific locations may represent 'endocytotic active zones'. The AP2 adaptor complex and dynamin appear to be localized to these 'endocytotic active zones'.

locations along the terminal [58*]. If the faster endocytotic pathway corresponds to a 'kiss-and-run' mechanism, the presence of elongated cisternae is somewhat surprising. However, the length of these cisternae appears to be attributable to the excess membrane built up from the block of dynamin function. For instance, after five minutes at 26°C, the collared pits of the slow endocytotic pathway elongate into branching tubule structures with electron-dense collars at branchpoints. An alternative explanation for the presence of elongated cisternae in the fast pathway is that dynamin may be required for the fission of synaptic vesicles in fast endocytotic pathways, as well as in slower clathrin-mediated pathways. This idea is supported by the finding that dynamin is required for rapid endocytosis in adrenal chromaffin cells (time constants are of the order of a few seconds) [64]. These electron micrographs, taken together with the subcellular localizations of α -adaptin and dynamin, suggest that two distinct endocytotic pathways can be simultaneously visualized within single synapses: fast endocytosis occurs near sites of exocytosis, while slower clathrin-mediated endocytosis occurs at endocytotic active zones, away from exocytotic active zones (Figure 1).

Concluding remarks

The past three years have seen a steady growth in the number of known mutations in synaptic proteins in *Drosophila*, and careful phenotypic characterization of these mutations should continue to provide useful *in vivo* insights into the mechanisms underlying synaptic

transmission. Genetic removal of syntaxin and n-syb in *Drosophila* indicates that neither is essential for the docking of synaptic vesicles and suggests that syntaxin, but not synaptobrevin, is absolutely required for neurotransmitter release. In terms of vesicle docking, it is possible that other SNARE proteins (such as synaptotagmin or SNAP-25) may compensate for the loss of syntaxin and synaptobrevin [12]. This possibility can be easily tested in *Drosophila* by the simultaneous removal of multiple SNAREs. However, if SNARE proteins do perform redundant functions, they must also play distinct, nonoverlapping roles, as deletions of syntaxin, n-syb, and synaptotagmin result in distinct mutant phenotypes (Table 1). Indeed, the specificity of these mutant phenotypes is not easily integrated into current biochemical models and suggests that it may still be too early to propose precise molecular models for synaptic vesicle exocytosis.

The recent analyses of α -*adaptin* and *shibire* mutants extend previous biochemical and ultrastructural studies by showing that clathrin-mediated endocytosis is essential for synaptic transmission. Furthermore, the ability to trap endocytotic intermediates using the *shibire* mutation has lent support to the view that fast and slow endocytotic pathways exist at distinct locations in the synapse. An emerging theme from these studies is that endocytosis of synaptic vesicles may almost be as spatially and temporally coordinated as exocytosis. To date, most studies on synaptic transmission in *Drosophila* have relied on previous biochemical and molecular analyses. Clearly, another promising and untapped approach is the identification of novel genes from functional genetic screens, which should facilitate the development of a more complete understanding of synaptic transmission.

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