TRANSMITTER RELEASE AT THE NEUROMUSCULAR JUNCTION

Thomas L. Schwarz

Program in Neurobiology, Children’s Hospital and Department of Neurobiology
Harvard Medical School, Boston, Massachusetts 02115, USA

I. Introduction
II. Physiological Properties of Transmitter Release
III. Experimental Advantages and Limitations of the Fly NMJ
   A. Ca\(^{2+}\) Measurements
   B. Dye Loading
   C. Alternative Measures of Exocytosis
IV. How Do Vesicles Fuse: Full Fusion or Kiss-and-Run?
V. Core Machinery of Exocytosis: Syntaxin, VAMP/Synaptobrevin, and SNAP-25
   A. Synaptobrevins in the Fly
   B. Tetanus Toxin as a Synapse-Silencing Reagent
   C. Syntaxin1
   D. SNAP-25 and SNAP-24
   E. SNARE Proteins Act Late in the Vesicle Cycle
   F. Summary of SNARE Proteins in Drosophila
VI. Vesicular ATPase and Membrane Fusion
VII. NSF and the Resetting of the SNARE Machinery
VIII. Synaptotagmin and the Regulation of Transmitter Release
    A. Synaptotagmin and Its Binding Properties
    B. Synaptotagmin Phenotypes at the NMJ
    C. Other Functions of Synaptotagmin
    D. Multiple Synaptotagmins in the Fly
    E. Summary of Synaptotagmin Function at the Fly NMJ
IX. Exocyst at the NMJ
X. Other Mutations of Proteins on the Target Membrane
   A. Rop/Unc-18/n-Sec1
   B. Unc-13 and Vesicle Priming
   C. CAST/ERK at the Active Zone
XI. Mutations in Peripheral Synaptic Vesicle Proteins
    A. Synapsin
    B. Cysteine String Protein and Hsc70
XII. Summary
     References

The mechanism of transmitter release at the Drosophila neuromuscular junction (NMJ) continues to be an area of active investigation in many laboratories. The undertaking has enhanced our knowledge of synaptic transmission and exocytosis by permitting in vivo tests of the significance of synaptic proteins. To explore the proteins that mediate exocytosis and synaptic transmission,
mutations in nearly two-dozen genes are available. Others that influence transmitter release indirectly, that is, mutations in endocytosis, development, or K$^+$ channels, are largely outside the scope of this chapter. From these studies a detailed picture emerges in which some proteins form a core apparatus for fusion while others contribute regulation and efficiency to the system. Yet other proteins may act in docking vesicles at active zones and in regulating their availability for fusion. Several interesting questions have been addressed. Is exocytosis primarily by the full fusion of vesicles with the plasma membrane or by “kiss-and-run”? Do the proteins that mediate fusion also account for the specificity of fusion at active zones? To what extent are the proteins of the synapse specialized and distinct from those that mediate exocytosis in nonneuronal cells? Are the soluble N-ethylmaleimide-sensitive factor attachment receptors (SNAREs) sufficient for synaptic vesicle fusion? How is the release of transmitter coupled to the action potential and its consequent influx of Ca$^{2+}$? In addition, the dissection of the fusion apparatus has given rise to the development of at least one reagent, tetanus toxin (TNT) light chain that is used as a transgene in *Drosophila* to block synapses and thereby to silence selected circuits. Thus, the investigation of synaptic mechanisms in *Drosophila* is helping us to understand synaptic cell biology in general and also provides tools with which to probe behavioral and developmental questions. This chapter will begin with a general discussion of the physiological properties of transmission at the NMJ and its cell biological underpinnings, and will then consider the individual proteins and their genetic analysis.

**I. Introduction**

The *Drosophila* neuromuscular junction (NMJ) has played a significant role in our understanding of neurotransmitter release. Genetic strategies have been used to test hypotheses concerning the function of proteins that had been implicated in this process by biochemistry or by yeast genetics. These studies have also led to physiological insights into the nature of the exocytotic event, distinctions among exocytotic pathways in neurons, and the mechanisms of modulating synaptic strength. The knowledge of the mechanism of transmitter release is also important as a basis for understanding how synaptic function develops and how synaptic activity can in turn shape development. Moreover, as investigators increasingly turn their attention to the functions of the central nervous system (CNS), including the circuits that govern behavior, synaptic proteins have gained further importance as a means of manipulating neurons within a circuit. What we know about these proteins in *Drosophila* is largely derived from studies of the NMJ. It is in this manner, rather than by the isolation of novel proteins in genetic screens, that *Drosophila* has influenced the field.
This chapter will first review our knowledge on transmitter release from a functional, physiological perspective, and will examine the nature of the exocytotic event. Thereafter, the proteins that mediate transmitter release will be reviewed with regard to their mutant phenotypes, and how the study of these phenotypes in *Drosophila* relates to the larger field of membrane traffic in neurons.

II. Physiological Properties of Transmitter Release

The release of neurotransmitter at the *Drosophila* NMJ is fundamentally akin to chemical transmission at vertebrate synapses. Neurotransmitter is packaged into synaptic vesicles and released into the synaptic cleft by the exocytosis of those vesicles. Single vesicles may fuse spontaneously, giving rise to unitary “quantal” events—miniature excitatory junctional potentials (mEJPs), also called miniature excitatory postsynaptic potentials (mEPSPs), or most colloquially just called “minis.” The chief distinction between *Drosophila* and vertebrate NMJs is that the fly motoneurons use glutamate as their transmitter rather than acetylcholine. In addition, whereas vertebrate NMJs are specialized to produce large responses, with each presynaptic action potential triggering an action potential in the muscle, the *Drosophila* NMJ gives rise to a subthreshold, graded response. In this regard, and in the use of glutamate as a transmitter, the fly NMJ is more akin to a central excitatory synapse in a vertebrate.

In response to an action potential, the opening of voltage-dependent Ca$_2^+$ channels at the presynaptic terminal causes a large increase in the probability of exocytosis. The consequent release of the contents of numerous vesicles gives rise to postsynaptic responses that represent the summed responses of individual quanta. If one is monitoring the voltage of the membrane (i.e., in “current clamp” mode), the altered voltage at the postsynaptic cell is called an EJP or sometimes EPSP. If instead, one is recording the current flowing through the channels (i.e., in voltage-clamp mode), the response is called an excitatory junctional current (EJC) or excitatory junctional postsynaptic current (EPSC). The latter mode of recording is generally preferable because the voltage change is not simply proportional to the number of channels opened or vesicles released, while the current flux under constant voltage is.

The delay between the arrival of the action potential to the presynaptic terminal and the fusion of the vesicles at the active zone is too brief to permit any large-scale movements of vesicles. Therefore, the vesicles are not released from the general pool at large in the nerve terminal, but only from a subset of vesicles that are immediately adjacent to the plasma membrane. These vesicles are often referred to as “docked” and sometimes are said to comprise the “readily releasable pool.” The concept of a docked vesicle is itself somewhat loose.
In some papers it refers only to an anatomical criterion of juxtaposition with the plasma membrane, while in others it implies a biochemical state in which vesicle proteins are interacting with proteins of the plasma membrane.

Although the details of Ca\(^{2+}\) influx and its spatial properties have not been studied in *Drosophila*, it is likely that, as in mammalian neurons, the release of neurotransmitter is triggered by microdomains of high Ca\(^{2+}\) that are formed near the mouth of voltage-dependent Ca\(^{2+}\) channels. From vertebrate studies it is known that resting Ca\(^{2+}\) in the cytosol is approximately 100 nM and that Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) may rise to several hundred micromoles within approximately 100 nm of the Ca\(^{2+}\) channel, while the channel is open. These microdomains—flashes of elevated Ca\(^{2+}\) that, as soon as the channel is closed, dissipate by diffusion into the larger volume of the terminal and by binding to cytosolic buffers—are formed in the immediate vicinity of docked, fusion-competent vesicles. Vesicles further away from the open Ca\(^{2+}\) channels will see much smaller increases in [Ca\(^{2+}\)] and will have low probabilities of fusion.

Which Ca\(^{2+}\) channels mediate the critical influx of Ca\(^{2+}\) in *Drosophila* presynaptic terminals? Although several genes coding for Ca\(^{2+}\) channels are present in the genome, the cacophony (cac) locus appears to be responsible for most, and perhaps all, of the Ca\(^{2+}\) influx that triggers transmitter release (Kawasaki *et al.*, 2000, 2004; Rieckhof *et al.*, 2003). These channels are localized at active zones and hypomorphic cac alleles cause reductions in transmitter release at the NMJ of third instar larvae. cac null animals die as embryos, presumably due to severe reductions in transmission. However, it might be unwise to presume that this gene mediates all Ca\(^{2+}\) influx at this or any synapse in *Drosophila*, as multiple Ca\(^{2+}\) channels can be present at mammalian synapses (Reid *et al.*, 2003).

Once inside the nerve terminal, multiple Ca\(^{2+}\) ions act cooperatively to trigger the fusion of a synaptic vesicle. This has been determined explicitly at the third instar NMJ by varying extracellular [Ca\(^{2+}\)] and observing the changes in the magnitude of transmitter release (Jan and Jan, 1976). The relationship between [Ca\(^{2+}\)] and vesicles released is nonlinear. The slope of this relationship on a log–log plot is equivalent to the Hill coefficient used by enzymologists as a measure of cooperativity in a biochemical reaction. Similarly, the slope of ln [Ca\(^{2+}\)] versus ln[vesicles released] is an indication of the number of Ca\(^{2+}\) ions needed to trigger vesicle fusion, or, more accurately, the cooperativity of Ca\(^{2+}\) in triggering fusion (Dodge and Rahamimoff, 1967). Because this method has been applied frequently, and not always accurately, some technical issues should be noted here. (1) The slope is related to the cooperativity of Ca\(^{2+}\) only at very low [Ca\(^{2+}\)], typically under 300 μM. At higher [Ca\(^{2+}\)] two factors can distort the measurement. Influx through Ca\(^{2+}\) channels may no longer be proportional to the extracellular [Ca\(^{2+}\)] and therefore the effective intracellular [Ca\(^{2+}\)] cannot be inferred from the external [Ca\(^{2+}\)]. Furthermore, the release apparatus itself may become saturated or partially limiting at high [Ca\(^{2+}\)]. In the extreme, the
quantal content of the response cannot be further increased by raising \( \text{Ca}^{2+} \) and the relationship has a slope of zero. (2) At the necessary low \([\text{Ca}^{2+}]\), the number of quanta released per impulse can be very low, particularly when examining a mutation that decreases release. These measurements frequently involve determining probabilities of release that are much less than one vesicle per action potential. In this range, it is important to distinguish spontaneous minis that arise coincidentally after the stimulus, from release evoked by the stimulus itself. Determining the frequency of spontaneous minis and subtracting their contribution from the observed responses is therefore a necessary correction. Both potential errors (saturation in higher \([\text{Ca}^{2+}]\) or the inclusion of spontaneous events) tend to flatten the measured dependency on \( \text{Ca}^{2+} \) and decrease the measured cooperativity. The best estimates of the cooperativity at wild-type third instar NMJs falls in the range of 3.5–4.5 \cite{Jan and Jan, 1976; Okamoto et al., 2005; Robinson et al., 2002}, a value consistent with similarly derived estimates of the number of \( \text{Ca}^{2+} \) ions needed for exocytosis at mammalian synapses. One further technical note: \( \text{Ca}^{2+} \) influx is not only a function of the extracellular \([\text{Ca}^{2+}]\) but it also depends on \([\text{Mg}^{2+}]\) ions, which can compete for access to \( \text{Ca}^{2+} \) channels. Thus, for a given \([\text{Ca}^{2+}]\), influx, and hence transmitter release, will be lower in a high \( \text{Mg}^{2+} \) saline, such as HL3 \cite{Stewart et al., 1994}, than in salines with less \( \text{Mg}^{2+} \) such as that of \cite{Jan and Jan, 1976}.

Most of the recordings made at the \textit{Drosophila} NMJ, either in third instar larvae or earlier, have been made from muscles 6 and 7 in the abdominal segments. These muscles are among the largest and most easily accessed if the larva is opened along the dorsal midline. However, there is at least one feature of this preparation that is not ideal and that must be kept in mind in experimental design and analysis. These muscles are innervated by two glutamate-gic neurons and each contributes to the EJP \cite{Hoang and Chiba, 2001; Lnenicka and Keshishian, 2000}. Damage to a preparation, improper adjustment of the stimulating voltage, high-frequency stimulation, or a mutant phenotype, may cause only one of the two axons to be stimulated and this will decrease the postsynaptic response. When working in high \( \text{Ca}^{2+} \) salines with large EJPs, it may be quite obvious when the amplitude of the response abruptly drops to a lower value, signaling that stimulation of one of the axons has failed. If one of the two axons is intermittently activated, the response will fluctuate between two amplitudes. In other conditions, where the quantal content is low and stimulus to stimulus variation is therefore large relative to the mean response amplitude, failure to conduct an action potential can be much more difficult to spot and may be confused with a true phenotype or with use-dependent fatigue of the synapse. Even more problematic is the possibility of branch-point failure—the failure of an action potential to invade all the terminal branches of the axons onto the muscle. Should this occur, the amplitude of the resulting change in EJP is unpredictable. The preference for recording from muscles 6 and 7 is largely
historical. In the future, it may be preferable to record from muscle 5, which receives input from only a single axon (Hoang and Chiba, 2001).

III. Experimental Advantages and Limitations of the Fly NMJ

The *Drosophila* NMJ has been invaluable for the analysis of mutations in synaptic proteins. The third instar larval preparation is among the easiest dissections in any organism. The muscle fibers are large and easy to impale and the nerve is easy to pick up and stimulate with a suction electrode. With an appropriate saline (Stewart *et al.*, 1994) it is possible to record for extended periods. This synapse, above all, gives an outstanding opportunity to record the detailed physiological properties of a great many mutations. The embryonic NMJ and first instar larval NMJ are considerably harder preparations to master. In particular, the dissections require patience and practice, but only this preparation permits recordings from strains with lethal mutations that will not develop beyond these stages. In both third instar and younger preparations, however, the individual quantal events are easily resolved from the noise and evoked responses can be studied in response to nerve stimulation. Individual muscles and their innervation are easy to identify and their synapses are stereotyped, showing comparatively little variation from animal to animal. Compared to neurons in the vertebrate CNS, these identified muscles provide admirable electrical properties and ease of access.

Nevertheless, some shortcomings of the preparation must be confessed, even while hoping that future work will overcome some of these obstacles.

A. **Ca**\(^{2+}\) **Measurements**

In *Drosophila*, it is not presently possible to measure directly the [Ca\(^{2+}\)] that arises in the immediate vicinity of synaptic release sites. Loading terminals with Ca\(^{2+}\) indicators, however, has permitted some measurements of the bulk rise in Ca\(^{2+}\) that occurs in the cytosol of the terminal after trains of action potentials (Dawson-Scully *et al.*, 2000). Methods for photolytic uncaging of Ca\(^{2+}\) in the cytosol have not been applied for the study of these synapses as a means of gaining more direct control of instantaneous changes in cytosolic Ca\(^{2+}\).

B. **Dye Loading**

The study of synaptic vesicle release has been restricted to two methods. The principle method is recordings from the postsynaptic cell whereby the
channels in the postsynaptic membrane serve as sensors for the transmitter released. The second method is the loading of synaptic vesicles with fluorescent dyes such as FM1-43 (Cochilla et al., 1999). The uptake of these dyes into reforming vesicles by endocytosis and the subsequent release of the dye can monitor vesicle fusion and endocytosis independently of the presence of neurotransmitter in those vesicles or the sensitivity of the postsynaptic cell (Chapter 7 by Kidokoro). However, the size of embryonic terminals and the vesicle pool within them has made it difficult to apply this method to study mutations with early lethal periods. Moreover, because the endings of larval motor neurons are typically embedded within the muscle cell, there are diffusion barriers for access to the synapse and the dye cannot be applied and removed as rapidly as it can, for example, in cultures of mammalian neurons. Therefore, while very precise rates of dye loading and unloading can be calculated with this method in mammalian systems, and dyes of different hydrophobicities can be compared (Richards et al., 2000), at the Drosophila NMJ this method cannot determine rates of endocytosis or exocytosis. Dye experiments, instead, are limited to the demonstration of the existence of cycling vesicles and measurements of the size of the cycling pool (Chapter 7 by Kidokoro).

C. ALTERNATIVE MEASURES OF EXOCYTOSIS

The Drosophila physiologist can also look with envy on some other preparations that are amenable to additional sophisticated methods for analyzing exocytosis. These include amperometry, capacitance measurements, and total internal reflection fluorescence (TIRF) microscopy.

Amperometry uses a carbon-fiber electrode to sense certain biogenic amines, chiefly dopamine, norepinephrine, and serotonin, and thereby measure the amount of biogenic amine that is released by the fusion of a vesicle independently of the sensitivity of the postsynaptic membrane (Zhou and Misler, 1995). At its best, amperometry can resolve partial release of vesicular contents. However, this method is not appropriate for glutamate and requires direct access to the synaptic cleft with the sensing electrode.

By patching directly onto endocrine cells or cells with large nerve terminals, and measuring the capacitance of the cell membrane, physiologists have been able to detect exocytosis, sometimes of single vesicles, in a manner that is altogether independent of the transmitter content of the vesicle. This means that the fusion of empty or partially loaded vesicles can be determined. In addition, capacitance measurements have been used to determine the resistance of the fusion pore and the time course of its opening at the onset of vesicle fusion (Beutner et al., 2001). However, because the Drosophila NMJ is typically very small and also embedded
in the muscle, it is not possible to patch directly onto the boutons for capacitance measurements.

*TIRF microscopy* employs fluorescent tags on or in synaptic vesicles and, by imaging vesicles within approximately 100 nM of the coverslip surface, can resolve the movements and fusions of single vesicles (Steyer et al., 1997). Again the geometry of the *Drosophila* NMJ prevents having release sites directly facing a coverslip, and therefore this technique has not been used in the fly. If it were to be adapted for *Drosophila*, it would probably require using cultured neurons and inducing them to synapse directly onto a coverslip.

### IV. How Do Vesicles Fuse: Full Fusion or Kiss-and-Run?

Before discussing the individual proteins that are involved in transmitter exocytosis, it is necessary to examine the larger question of precisely how the transmitter leaves the synaptic vesicle and enters the synaptic cleft. This has been a long-running controversy in cell biology and neuroscience, dating back at least 30 years (Ceccarelli and Hurlbut, 1980; Heuser and Reese, 1973). The issue can be summarized as follows. Classical exocytosis, as observed in many cell types, involves the complete fusion of a vesicle with the plasma membrane. The vesicle flattens out and is incorporated into the plasma membrane. Its contents are completely disgorged into the extracellular space. At a nerve terminal, where vesicles must be recycled rapidly for reuse, this exocytosis would be followed by endocytosis, probably via clathrin, to recover the membrane and its proteins into a new synaptic vesicle (Chapter 7 by Kidokoro). An alternative model, often called kiss-and-run, invokes a mechanism in which the vesicular membrane does not completely merge with and flatten out onto the plasma membrane. Instead, a brief, partial fusion is envisioned, in which a transient fusion pore is opened between the vesicle lumen and the extracellular space. The vesicle is reformed by the reversal of this process and the closing of the fusion pore. If the pore is open very briefly, it is possible that transmitter will only partially be unloaded. A hallmark of the kiss-and-run process is that fusion is not complete and that classical endocytosis is not needed to remake a synaptic vesicle. The literature from mammalian systems, for and against the existence of a kiss-and-run mechanism, is too extensive to be reviewed here (An and Zenisek, 2004; Jarousse and Kelly, 2001). On several points the field has not yet reached a consensus, but some points are generally accepted: (1) classical exocytosis and endocytosis are likely to exist at most chemical synapses; (2) partial release of transmitter due to incomplete unloading appears to occur, at least some of the time, in the case of large dense-cored vesicles released from nonsynaptic sites; and (3) if kiss-and-run
does occur from small clear vesicles at synapses, it may occur only under certain circumstances or alongside classical exocytotic events.

At the *Drosophila* NMJ, the issue of kiss-and-run has surfaced and has provoked some debate (Dickman et al., 2005; Verstreken et al., 2002). There is no doubt that full fusion and classical endocytosis occur and account for many of the fusion events at this synapse. The proteins of the classical endocytotic pathway are present at presynaptic endings and mutations in a clathrin adaptor protein, Lap-180, can alter the size of synaptic vesicles (Bao et al., 2005; Zhang et al., 1998a). Mutations in *shibire* (*shi*) have been extremely informative. This gene encodes the protein dynamin, a component of the classical endocytotic pathway that severs nascent vesicles from the plasma membrane. As discussed elsewhere in this volume (Chapter 7 by Kidokoro), *shi* temperature-sensitive mutations, at a nonpermissive temperature, can cause the run down of a synapse on stimulation. Vesicles that fuse to the plasma membrane are trapped there until eventually the releasable pool of vesicles is completely transferred to the plasma membrane (Koenig and Ikeda, 1989; van de Goor et al., 1995). These observations are consistent with classical exocytosis serving as the dominant form of transmitter release at the *Drosophila* NMJ. The coexistence of kiss-and-run, however, was reported from an examination of mutations in other proteins in the classical endocytotic pathway, Endophilin (Endo) and Synaptojanin (Synj) (Verstreken et al., 2002, 2003). The response of *endo* and *synj* mutants to repeated stimulation at 10 Hz is quite different from that of a *shi* mutant. Whereas the amplitude of the EJP declines in *shi* until a negligible response remains, amplitudes in *endo* and *synj* larvae decline only partially and then reach a plateau level that can be sustained, seemingly indefinitely. During this plateau many more vesicles are released than were originally present in the terminal, and thus, there must be a means to recycle and reuse synaptic vesicles even in the absence of these proteins. Arguing that these mutations block the classical recycling pathway, the authors concluded that the plateau phase must be maintained by another pathway and in particular by kiss-and-run. Further analysis of these mutations, however, has demonstrated that they do not completely block classical endocytosis, but rather diminish the maximum rate of endocytosis that can be achieved at the NMJ (Dickman et al., 2005). The residual slowed pathway has the properties of classical endocytosis, including the ability for vesicles to be loaded with the dye FM1–43 (Chapter 7 by Kidokoro) and a dependency on Dynamin/Shi. The residual slow pathway is sufficient to account for the ability of *synj* and *endo* terminals to sustain a reduced level of transmitter release even under high-frequency stimulation. Thus, although it is not possible to completely exclude the existence of kiss-and-run at this synapse, there is presently no evidence for its occurrence and the full-fusion pathway is sufficient to explain the physiology of the system to date, including the absolute requirement for Dynamin/Shi for sustained vesicle cycling.
A wealth of data from yeast to mammals has demonstrated the existence of a core machinery for intracellular membrane fusion. Versions of the proteins that form this machinery are present at synapses and can also be found on vesicles that shuttle between the endoplasmic reticulum (ER) and Golgi in yeast, and in all membrane trafficking compartments of every eukaryotic cell. In the effort to understand the workings of these proteins, the Drosophila NMJ has proven valuable. It has the advantages, of course, of having the ability to examine mutations in the genes encoding for these proteins. However, it also provides the capacity to resolve single vesicle fusions with rapid kinetics by electrophysiological means and to examine the ability of vesicles to find and dock at their target membranes by electron microscopy. Additionally, the manipulation of one of these proteins by the expression of tetanus toxin (TNT) is now used extensively to alter the activity of circuits within the fly brain (Heimbeck et al., 1999; Kaneko et al., 2000; Martin et al., 2002; Suster et al., 2003; Sweeney et al., 1995). For this reason, the role of the fusion complex will be examined here in detail.

The proteins of the core complex are often referred to as soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins (Sollner et al., 1993b) and the prevailing model, compiled from experiments in yeast, in synaptic preparations, and in vitro fusion assays, is that these proteins, by binding to one another, can form a bridge between a vesicle and its target membrane. Moreover, the energy released by the formation of this complex may provide the driving force for the actual fusion of the two membranes (Chen and Scheller, 2001). Fusion has been shown to require complementary proteins on either side of the reaction, that is, on both the vesicle and target membranes. These are sometimes referred to as v- and t-SNARES, although the distinction as to who is the vesicle and who the target is sometimes not so clear. At the synapse, the t-SNARES (found principally on the plasma membrane) are called Syntaxin and SNAP-25. The v-SNARE (that resides chiefly on the synaptic vesicles) is called either Synaptobrevin or VAMP. The complex that forms between these proteins consists of a four-stranded coiled coil. Two of the component strands are contributed by SNAP-25 and one each by the other proteins. The complex they form is exceptionally stable and it has been hypothesized that vesicles docked at the plasma membrane can form a loose complex first, which, at the time of vesicle fusion, is allowed to “zipper closed.” This zipperring drives the two membranes together (Chen and Scheller, 2001; Lin and Scheller, 2000). In addition, it was noted that different trafficking compartments within the cell can have different isoforms of these proteins and that not all isoforms will function in conjunction with one another (McNew et al., 2000). Thus, it has been hypothesized that the cognate pairing of v- and t-SNAREs may provide the
specificity that allows a vesicle to fuse selectively with its appropriate target membrane.

There have been several key questions regarding the function of this core complex that have been investigated at the fly NMJ. (1) Are these proteins essential for all types of synaptic vesicle fusion? (2) Is an individual SNARE used only for synaptic vesicle fusion or is it used for other types of exocytosis? (3) Are SNAREs necessary for an early step in transmitter release, particularly for the docking of vesicles at the active zone, or only for the final membrane fusion step? (4) Do the SNAREs show specificity in their ability to support synaptic vesicle fusion? Some or all of these questions have been addressed with mutations in the genes coding for each of the v- and t-SNAREs.

A. SYNAPTOBREVINS IN THE FLY

There are at least two isoforms of Synaptobrevin/VAMP in *Drosophila*. One, called Synaptobrevin (Syb), is ubiquitously expressed in the organism (Chin et al., 1993; DiAntonio et al., 1993a; Sudhof et al., 1989). Mutations in the *syb* gene are embryonic lethals, causing arrest of development at what is probably the time when the maternally contributed *syb* runs out. Clones of *syb* null mutants are cell lethal in the eye (Bhattacharya et al., 2002). Thus, although there have been no direct assays of membrane traffic in these cells, it seems likely, based on its phenotype and homology to Synaptobrevins in other species, that Syb mediates the constitutive trafficking of proteins to the cell surface. Without this traffic, cells cannot grow or divide.

The second Synaptobrevin isoform is called neuronal-Synaptobrevin (n-Syb) and it is detected exclusively in the nervous system, where it appears to be highly concentrated at all synapses (Deitcher et al., 1998; DiAntonio et al., 1993a). The first analysis of *n-syb* function in *Drosophila* was not via mutations in the gene, but by the expression of a transgene encoding the light chain of TNT (Broadie et al., 1995; Sweeney et al., 1995). TNT is a product of a Clostridial bacterium, and the light chain of TNT is a protease that selectively cleaves certain isoforms of Synaptobrevin (Pellizzari et al., 1999). In *Drosophila*, it cleaves n-Syb, but not Syb. Neuronal expression of TNT did not impair the differentiation of motor-neurons or their ability to extend axons and form synapses, but embryos were paralyzed and could not hatch (Sweeney et al., 1995). Physiological recordings from embryonic NMJs uncovered a surprising result: EJPs could not be evoked in the TNT-expressing flies, but spontaneous minis were still observed. The former finding was, of course, consistent with the hypothesis that n-Syb is the essential v-SNARE for the fusion of synaptic vesicles, but the persistence of minis was confounding. This phenomenon has been studied more intensively in null alleles of *n-syb*, which circumvent some potential problems of TNT expression—that
some protein may persist intact or that the cleaved products may retain some activity. The phenotype of the n-syb nulls, however, was identical to that observed on TNT expression. Null mutations lacking n-syb develop normally but are paralyzed and do not hatch. Even under conditions where evoked EJPs should be very large (high Ca\textsuperscript{2+} or the presence of K\textsuperscript{+} channel blockers) no EJP was seen. Minis, however, were normal in size and present at approximately 25% the frequency of wild type (Deitcher et al., 1998). These findings are novel because they distinguish the mechanistic requirements for spontaneous and evoked vesicle fusions. If evoked release were merely the same process as a mini, but with increased probability and synchronized, the two types of fusion would be equally dependent on the same SNARE proteins.

Do the remaining minis fuse via a novel mechanism, completely independent of SNARE proteins? This is unlikely because, as discussed later, minis are reported to be completely absent in flies lacking the t-SNARE Syntaxin. However, SNARE-dependent fusion requires a SNARE on the vesicle membrane as well (Nichols et al., 1997). What then is the mechanism for the release of these minis? One possibility is that they employ the other v-SNARE, Syb. Another is that a t-SNARE, perhaps Syntaxin, is partially localized on synaptic vesicles and can substitute for n-Syb in the formation of the core complex. There is precedent for fusion proceeding, albeit poorly, by means of t-SNARE–t-SNARE complexes (Nichols et al., 1997). Whatever this fusion pathway might be, it is not capable of the fast, synchronous fusions that form an EJP. In a detailed study of these residual events (Yoshihara et al., 1999), it was determined that their frequency can be modulated by cytosolic Ca\textsuperscript{2+}, but the elevated Ca\textsuperscript{2+} must be sustained. Thus, in an n-syb null mutant, the frequency of minis could be increased up to 17-fold by a steady state depolarization of the terminal, a tetanic stimulation of the nerve, or direct Ca\textsuperscript{2+} influx via an ionophore. Thus, n-syb mutant terminals retain a Ca\textsuperscript{2+}-sensor that can couple to the remaining release apparatus. The inability of Ca\textsuperscript{2+} influx during a single action potential to have a similar effect could indicate that the triggering is too slow to be activated by a transient change in local Ca\textsuperscript{2+}, or that the vesicles are no longer in the immediate vicinity of the open Ca\textsuperscript{2+} channels.

Another fascinating aspect of these mutant terminals is that the release apparatus is no longer modulated by cAMP (Yoshihara et al., 1999). In wild-type larvae, cAMP, via Protein Kinase A, has two actions at the synapse. One depends on extracellular Ca\textsuperscript{2+} and likely involves the modulation of Ca\textsuperscript{2+} channels. The other is independent of extracellular Ca\textsuperscript{2+} and likely involves a modulation of the release apparatus that increases mini frequency. This Ca\textsuperscript{2+}-independent action was absent from n-syb larvae (Yoshihara et al., 2000) and thus the pathway downstream of cAMP must pass through this particular v-SNARE. Another interesting feature of the n-syb phenotype was noted in viable hypomorphic alleles (Stewart et al., 2000): a shift in the apparent cooperativity of Ca\textsuperscript{2+}
ions in triggering release, as measured by systematically altering the extracellular 
$[\text{Ca}^{2+}]$. This finding may reflect the fact that $\text{Ca}^{2+}$, having bound to its sensor, 
must act via the SNARE complex.

Are v-SNAREs specific for particular types of membrane traffic? The specificity of SNARE pairing is a key element of the hypothesis that SNAREs direct vesicles to the correct target membrane. Through the availability of null mutations in both $\text{syb}$ and $n$-$\text{syb}$, and of transgenes to drive expression of each, it has been possible to ask whether they are either interchangeable or selective in their particular roles, that is, in cell survival and growth versus synaptic transmission (Bhattacharya et al., 2002). The expression of $n$-$\text{syb}$ in the developing $\text{syb}$ null eye could in fact rescue its development and the expression of $\text{syb}$ could partially restore the EJP at an $n$-$\text{syb}$ null NMJ. Moreover, these two v-SNARES have behaved identically in biochemical assays of complex formation (Niemeyer and Schwarz, 2000). Thus, Synaptobrevins may or may not have selectivity for fusion with the plasma membrane instead of with intracellular organelles. However, they cannot account for the selective fusion of synaptic vesicles at active zones, as opposed to the fusion of other vesicles that deliver proteins to other sites on the cell surface.

B. Tetanus Toxin as a Synapse-Silencing Reagent

As mentioned earlier, the light chain of TNT can cleave n-Syb and it does so with great specificity as well as great efficiency: little or no intact n-Syb remains when TNT expression is driven throughout the nervous system (Sweeney et al., 1995). There has therefore been great interest in selectively expressing TNT in subsets of neurons to silence their output (Martin et al., 2002). This method has been used to establish the functional importance of neurons for such behaviors as feeding and locomotion (Heimbeck et al., 1999; Kaneko et al., 2000; Suster et al., 2003; Sweeney et al., 1995). However, some important caveats need to be considered, because it is not possible in the CNS to determine directly whether or not TNT expression in the selected neurons is effective in preventing transmitter release. Instead, there is a presumption, not necessarily accurate, that those cells will be affected in the same manner as the well-studied motoneurons. We do not know, however, that every neuron contains its target, n-Syb, although it is certainly widespread (DiAntonio et al., 1993a). Moreover, since overexpression of Syb, which is not cleaved by TNT, can substitute, at least in part, for n-Syb (Bhattacharya et al., 2002), it is possible that high levels of Syb or a related SNARE might sustain transmission in subpopulations of CNS synapses. Similarly, even at the NMJ, TNT does not abolish transmitter release. It only abolishes the efficient, synchronous release of transmitter in response to individual action potentials. Spontaneous minis can therefore be expected to remain
in the CNS and any sustained electrical activity, such as high-frequency trains of action potentials, or sustained depolarizations in nonspiking neurons, such as photoreceptors, would be expected to increase the release of transmitter despite the removal of n-Syb. Finally, there are many forms of transmitter release that may differ mechanistically from what we have studied in detail at the glutamatergic motoneuron synapses. The efficacy of TNT in preventing the release of aminergic vesicles or dense-cored peptidergic vesicles is only now being examined. Release of transmitter from nonvesicular pools, for example, from plasma membrane transporters running “backward” is presumed to be TNT-insensitive (Yang and Kunes, 2004). Finally, although loss of n-Syb does not cause gross abnormalities in the development of neurons, the possibility remains that the effects of TNT will not be exclusively on the fusion of synaptic vesicles. TNT may alter the release of signaling molecules, or the surface expression of some receptors. In summary, although TNT expression is a highly valuable tool for neurogenetics, it may not silence all forms of synaptic transmission or be devoid of developmental consequences.

C. SYNTAXIN1

Of the SNAREs that are localized to the plasma membrane, the most attention has been given to Syntaxin. Although Syntaxin isoforms are present on many compartments within the cell, a particular Syntaxin, called Syntaxin1 (Syx1) in Drosophila, appears to be the homolog of the Syntaxins found on the plasma membrane in mammalian cells (Schulze et al., 1995). Genetic analysis of this protein at the synapse is complicated by the fact that Syx1 is not exclusively synaptic. Mutations in syx1 are cell lethal, suggesting that it is important in cell growth and division, like the v-SNARE syb (Schulze et al., 1995; Stowers and Schwarz, 1999). Maternal germ-line mosaics of null syx1 alleles are infertile due to arrest of oocyte development. Similarly, mosaics of weaker alleles give rise to embryos with defects in cellularization, a process that involves a large increase in membrane surface area (Burgess et al., 1997). These phenotypes attest to the essential role that Syx1 plays in membrane traffic to the cell surface.

However, due to maternally contributed protein and mRNA, homozygous null mutant embryos from heterozygous parents can develop until late stages of embryogenesis before dying (Broadie et al., 1995; Burgess et al., 1997; Schulze et al., 1995). These embryos have abnormalities indicative of secretory defects in nonneuronal cells and also mild anatomical abnormalities in their central and peripheral nervous system. Nevertheless, the maternal contribution appears to be just barely enough that, at least in most segments, motoneurons are born and can extend axons to the appropriate muscle targets, but not enough for synapses to function properly (Schulze et al., 1995). Several papers have appeared in which
the electrophysiological and developmental properties of these synapses are described (Broadie et al., 1995; Featherstone and Broadie, 2002; Saitoe et al., 2001, 2002; Schulze et al., 1995), and their findings are not always in agreement. The differences are likely, at least in part, to be due to the fact that these embryonic synapses are poised at a threshold where the maternally contributed Syx1 is disappearing. Therefore, the age of the embryo, the position of the muscle, and other unknown variables, may cause some recordings to be made from synapses that still contain low levels of Syx1, while others have so little Syx1 that the synapse never formed or formed and then began to retract. In no case has an EJP been evoked from a homozygous null syx1 embryo, consistent with a requisite role for Syx1 in transmitter release (Broadie et al., 1995; Saitoe et al., 2001; Schulze et al., 1995). It has also been reported that, contrary to initial reports (Broadie et al., 1995), quanta are not released from the syx terminals by the application of hypertonic sucrose solutions (Aravamudan et al., 1999). Hypertonic sucrose has been used in mammalian systems to trigger the release of vesicles from nerve terminals by an unknown mechanism that bypasses the normal requirement for Ca$^{2+}$ influx. This finding again emphasizes the importance of Syntaxin for the fusion of synaptic vesicles, and further suggests that sucrose triggers release in a SNARE-dependent manner. However, one group has observed spontaneous minis at early stages of synapse formation in syx1 mutants (perhaps while some Syx1 still remains). Subsequently this group observed a large decrease in the sensitivity of the muscle membrane to glutamate and an absence of glutamate receptors clusters. These findings were interpreted as showing that minis are needed to form or preserve glutamate receptors clusters opposite to active zones (Saitoe et al., 2001, 2002). Others have observed normal levels of glutamate sensitivity and the absence of minis (Featherstone and Broadie, 2002). These findings underscore the difficulty of analyzing the function of a protein in transmitter release when that protein is also required for constitutive membrane traffic and hence for the viability of the cell and the formation of the synapse.

Biochemical studies of the H3 domain of mammalian Syntaxin1 indicate that it can bind to several proteins other than Synaptobrevin/VAMP and SNAP-25, the proteins of the core complex. In particular, Syntaxin1 can bind to the protein known as n-Sec1 or Munc-18 in mammalian systems and encoded by ras opposite (rop) in Drosophila. This is a very high affinity interaction, and its function is not yet clear. However, n-Sec1 is not present in the core complex, and therefore, one hypothesis is that it holds Syntaxin in a closed state that prevents it from associating with the other SNAREs (Dulubova et al., 1999). Mutations in rop are embryonic lethal and, like syb or syx1 mutations, have defects in nonneuronal structures, suggesting a requirement in constitutive membrane trafficking (Harrison et al., 1994). This phenotype (and the phenotype of sec1 mutations in yeast; Novick et al., 1980) is not compatible with n-Sec1 functioning solely as a negative regulator of release: its phenotype indicates a decrease in secretion,
not an increase. Syntaxin also binds to the vesicle protein Synaptotagmin (see in a later section) and may bind to a region of mammalian Ca\(^{2+}\) channels (Bezprozvanny et al., 2000; Leveque et al., 1994). Attempts have been made to understand the physiological significance of these interactions in *Drosophila* by using point mutations that selectively alter one or another of the protein–protein interactions (Fergestad et al., 2001; Wu et al., 1999). Portions of these studies, however, have been challenged on the basis that the mutations used do not selectively or sufficiently alter the interactions in question (Matos et al., 2000).

D. SNAP-25 AND SNAP-24

The other plasma membrane t-SNARE, SNAP-25, has been particularly difficult to study in *Drosophila*, in part because the gene is located within heterochromatin and is composed of multiple exons that are small and widely spaced (Risinger et al., 1997). In addition, it is partially redundant with a homolog protein called SNAP-24 (Niemeyer and Schwarz, 2000; Vilinsky et al., 2002). The first allele of *Snap25* to be isolated was a temperature-sensitive paralytic mutation that resulted in a single amino acid change in the first amphipathic coil of SNAP-25 (Rao et al., 2001). Biochemical analysis indicated that this protein remained capable of forming SNARE complexes. The only temperature-sensitive defect that was detected in this mutant was in the little-understood phenomenon of SNARE complex multimerization—the presence of higher molecular weight complexes of SNARE proteins on SDS gels. These presumed multimers of the SNARE complex were less stable if SNAP-25 contained the point mutation. *In vivo*, the temperature-sensitive allele had the unexpected property of increasing EJP amplitude at the permissive temperature, perhaps reflecting an enhanced ability of the mutant protein to proceed toward fusion (Rao et al., 2001). At elevated temperatures, however, transmission was reduced relative to wild type, which is the likely cause of the temperature-sensitive paralysis and suggests that the multimerization of SNAREs may be important to their function *in vivo*.

The isolation of null alleles of *Snap25* offered an additional surprise: homozygotes could survive through pupal stages and synaptic transmission at the larval NMJ was largely unaffected by the absence of the protein (Vilinsky et al., 2002). This phenomenon is likely to be explained by the presence of the closely related t-SNARE SNAP-24. SNAP-24 is less abundant than SNAP-25 within the nervous system and is more abundant in nonneuronal tissues, suggesting that it is primarily concerned with constitutive membrane traffic. Instead, SNAP-25 predominates at nerve terminals (Niemeyer and Schwarz, 2000). However, in the complete absence of SNAP-25 protein (which is not the case in the temperature-sensitive *Snap25* allele) SNAP-24 can replace SNAP-25 (Vilinsky et al., 2002). In biochemical studies, SNAP-24 and SNAP-25 were
indistinguishable in their shared ability to form SNARE complexes with Syntaxin and n-Syb, consistent with their interchangeability during transmitter release (Niemeyer and Schwarz, 2000). In addition, both isoforms could form SNARE complexes with Syntaxin and Syb, the likely complex involved in constitutive membrane traffic. Thus, as discussed earlier for n-Syb and Syb, the t-SNAREs SNAP-24 and SNAP-25 are interchangeable. Specificity of cognate recognition by SNARE proteins cannot explain the distinctions in membrane targeting between synaptic vesicles that fuse at active zones and constitutive vesicles that fuse elsewhere on the surface.

E. SNARE PROTEINS ACT LATE IN THE VESICLE CYCLE

The study of n-Syb (as well as Syntaxin, see in a later section) has been useful in determining the step at which SNAREs act, that is, whether in the docking of vesicles at their target membranes, in the priming of them for fusion, or in the fusion step itself. For these questions of general cell biological importance, the Drosophila NMJ has several advantages. Not only are there unambiguous null alleles for SNARE proteins, but there is also a well-defined target membrane that normally contains a population of docked vesicles that are visible by electron microscopy. It is not clear just how this pool, defined as “docked” by anatomical criteria, correlates with the biochemist’s model of “docked” vesicles, which presumes an association of the vesicle and target membranes via protein–protein interactions. Nonetheless, in the case of syx1 null alleles or in nerve endings expressing TNT, there is no detectable loss of synaptic vesicles in close juxtaposition to the active zone membrane. On the contrary, the number of docked vesicles appears to be increased (Broadie et al., 1995). These findings indicate that synaptic vesicles can find nerve terminals, cluster around active zones, and dock at the active zone membrane in the absence of the SNARE proteins. The absence of evoked release, in contrast, argues strongly for a block at a late stage in the process, most likely in fusion itself. The intermediate steps that are hypothesized to occur in order to make a vesicle ready for rapid fusion after it contacts the plasma membrane (collectively referred to as priming), are more difficult to judge. The presence of minis in n-syb null alleles, and their ability to fuse in response to sustained elevation of Ca\(^{2+}\), may indicate that some vesicles are primed for fusion, but their slower rate of fusion indicates that they are not as competent for fusion as vesicles in wild type.

F. SUMMARY OF SNARE PROTEINS IN DROSOPHILA

In summary, null alleles are available for each of the v- and t-SNAREs that function at the synapse, syx1, n-syb, and Snap25, as well as for the close
homolog syb that principally functions in nonsynaptic traffic to the plasma membrane. In addition, TNT can selectively cleave n-Syb and thereby prevent the evoked release of transmitter. By selective expression in particular cell types, TNT may be a powerful means to silence the output of a cell, although some caution is needed in extrapolating from the NMJ to the CNS. From studies of the fly NMJ we have learned that mutations in SNAREs can differentially affect evoked release and minis, suggesting mechanistic differences in the nature of vesicle fusion in these two processes. Because morphologically docked vesicles persist in these mutants, the SNAREs are likely to act specifically in a late step of the vesicle cycle, most probably in fusion itself. In addition, although there are distinctions between the Syb isoforms that normally predominate in constitutive versus synaptic membrane traffic, those isoforms can substitute for one another in functional assays. In addition, they show no biochemical specificity in their ability to complex with one another. Moreover, a single Syx isoform is used in both pathways. Therefore, SNARE pairing does not provide specificity for targeting these classes of vesicles to their particular target membrane at the active zone.

VI. Vesicular ATPase and Membrane Fusion

The demonstration that SNARE proteins function in a late stage of exocytosis raises the question as to whether they are the sole requirement for membrane fusion. There have been in vitro studies to demonstrate that SNAREs by themselves can promote the fusion of lipid vesicles in vitro (Weber et al., 1998), but this does not necessarily mean that this minimal reconstituted system represents the normal in vivo mechanism. In this context, one of the most intriguing studies using the Drosophila NMJ (Hiesinger et al., 2005) proposes that a subunit of the vesicular ATPase, an abundant and required protein in the synaptic vesicle membrane, also functions at a late step of exocytosis, perhaps by forming a fusion pore bridging the membranes. The vesicular ATPase is the proton pump responsible for the acidification of the vesicle lumen at the expense of ATP. As such it provides, in the form of a proton gradient, the energy that drives the uptake of transmitter into vesicles. A hypothesis is that at least one of the proteins in this large complex (the \( v100-1 \) subunit of the V\(_0\) complex) functions directly in membrane fusion.

This model has origins in studies of vacuolar fusion in yeast (Bayer et al., 2003; Peters et al., 2001) wherein it was found that a particular subunit of the vacuolar ATPase, Vph1p, was necessary in assays of vacuole fusion with one another in vitro. This ATPase comprises two large complexes, one that hydrolyzes ATP and another that forms a proteolipid pore through which protons can flux. The Vph1p subunit is in the latter, the V\(_0\) complex. However, the particular
mutations studied did not block proton transport, the canonical function of the V₀ complex. Rather, the defect in the yeast assay was specifically in fusion, at a step after the requirement for the SNARE proteins. It was hypothesized that the large Vph₁p protein formed a bridge between two vesicles that were linked by SNAREs and that this bridge could progress into a proteinaceous pore that subsequently would resolve into full fusion of the membranes.

In this context, it was striking that mutations in the Drosophila homolog of the same large subunit were isolated in a phototaxis screen designed to identify mutations of synaptic transmission (Hiesinger et al., 2005). The subunit, called v₁₀₀₋₁, is concentrated at synapses, and null mutations in the v₁₀₀₋₁ gene showed a sevenfold reduction in EJP amplitude at the embryonic NMJ. Minis were present and of normal amplitude, although reduced in frequency. The presence of some minis indicates that some transmitter loading and some fusion can occur in these embryos, perhaps because of a partially redundant second isoform. But why is the EJP so small? Is it because of a block in fusion or because a lack of proton transport has generated empty vesicles? The evidence favors the former because FM₁-₄₃ dye loading was below normal levels (Hiesinger et al., 2005). Moreover, both in yeast and in the fly, the v₁₀₀₋₁ subunit can bind to t-SNAREs (Hiesinger et al., 2005; Peters et al., 2001). Thus, while the precise role of this ATPase subunit remains uncertain, the hypothesis that it interacts with t-SNAREs to form a fusion pore, or to initiate membrane fusion is an attractive possibility.

VII. NSF and the Resetting of the SNARE Machinery

NSF, a factor that support in vitro vesicle trafficking in cell extracts (Block et al., 1988), is the key element of a multimeric protein that includes six NSF subunits and six subunits of an associated protein called alpha-SNAP. NSF can bind to SNARE complexes and, through the catalysis of ATP, can cause them to dissociate (Sollner et al., 1993a). Originally, it was thought that this ATP-dependent step might represent the fusion reaction itself, but this model has been rejected because fusion in many systems is ATP-independent, and because SNAREs in vitro can stimulate membrane fusion without NSF (Weber et al., 1998). Models (Lin and Scheller, 2000; Schwarz, 1999) place NSF action after fusion as a means of undoing SNARE complexes so that v- and t-SNAREs can be separated from one another and recycled to their proper compartments. The formation of the SNARE complex is so energetically favorable that energy (provided by the ATPase activity of NSF) is required to pull the SNAREs apart and free them for another round of vesicle fusion. Separated, the SNAREs are in a state with high potential energy and that energy can be harnessed by the
cell when SNARE complexes subsequently reform and thereby drive membrane fusion.

The comatose (comt) gene in Drosophila encodes an NSF homolog (Pallanck et al., 1995). The study of comt permitted a crucial genetic test of NSF function in vivo. The classic comt alleles are temperature-sensitive paralytics (Siddiqi and Benzer, 1976). These alleles are hypomorphic mutations of Nsf1, one of two NSF homologs in the fly (Pallanck et al., 1995). Consistent with the model described above, when shifted to the nonpermissive temperature, comt flies accumulate SNARE complexes that reside primarily on the plasma membrane (Tolar and Pallanck, 1998). Using electron microscopy it is observed that these terminals accumulate vesicles, including a population that is docked at the active zone by morphological criteria, suggesting a defect in the priming of vesicles for transmitter release (Kawasaki et al., 1998). A likely explanation (Littleton et al., 2001) is that each round of vesicle fusion in a comt mutant creates SNARE complexes on the plasma membrane that cannot be dissociated and that, over time, the cycling vesicle pool is depleted of usable, free, SNAREs. At this point, the vesicles can no longer fuse with the membrane and are therefore trapped in a docked but fusion-incompetent state.

At adult NMJs on the dorsolateral flight muscles, the electrophysiological phenotype of comt mutants is wholly consonant with this model. At the permissive temperature the EJP is normal but, at the nonpermissive temperature, there is a progressive decline in EJP amplitude. This likely reflects the loss of free SNAREs and hence the decline in releasable vesicles (Kawasaki et al., 1998). At the third instar larval NMJ, however, comt does not have a detectable phenotype (Golby et al., 2001; Mohtashami et al., 2001). This can be explained by the fact that the Nsf1 gene that is mutant in comt appears to be predominantly expressed in the adult nervous system, while NSF2 is likely to play an equivalent role at the larval NMJ (Stewart et al., 2002). Mutations in Nsf2 are early lethals, perhaps reflecting the importance of this isoform for membrane traffic in non-neuronal cells (Golby et al., 2001). To study the physiological consequences of disrupting NSF2 at the larval NMJ, a dominant negative approach has therefore been taken (Stewart et al., 2002, 2005). Expression of a dominant negative NSF2 in larval neurons has the expected consequence of causing use-dependent rundown of the amplitude of the EJP. However, it also causes a substantial overgrowth of the synapse, even at early developmental stages, and some axonal misrouting. The trafficking defect behind these developmental phenotypes is not yet known.

The relationship of the two Nsf genes in Drosophila is not yet completely understood. However, the apparent importance of NSF2 in nonneuronal embryonic tissues as well as at larval synapses provides another example of membrane trafficking proteins that are shared between synaptic and nonsynaptic pathways.
There are no known differences in the SNARE proteins at adult versus larval synapses, and so it seems unlikely that the two NSF isoforms will differ in their substrate specificities.

VIII. Synaptotagmin and the Regulation of Transmitter Release

A. Synaptotagmin and Its Binding Properties

Ever since the identification of Synaptotagmin as a major synaptic vesicle protein and the determination of its sequence (Perin et al., 1990), Synaptotagmin has been the focus of intense study. The biochemical properties of mammalian Synaptotagmin have been studied in great detail (Chapman, 2002; Li et al., 1995; Rizo and Sudhof, 1998; Sudhof and Rizo, 1996), and some of these properties have been confirmed for Drosophila. The hallmark of the Synaptotagmin protein family is an N-terminal transmembrane domain, followed by a large cytoplasmic domain containing two C2 domains in tandem. The C2 domain is a much-studied motif also present in isoforms of Protein Kinase C and Phospholipase A2. In these proteins the C2 domain binds Ca^{2+} and causes a conformational change in the protein that triggers its translocation to the membrane (Nalefski et al., 2001). The C2 domains of Synaptotagmin also bind Ca^{2+} via a set of conserved negative charges that are clustered at one end of the structure (Shao et al., 1996, 1998). In addition, C2 domains of Synaptotagmin also bind phospholipids and phosphatidyl inositol (Li et al., 1995; Zhang et al., 1998b). These binding properties strongly suggest an important role for Synaptotagmin in the regulation of transmitter release. Synaptotagmin is the best candidate for being the sensor for cytosolic Ca^{2+} that triggers the release of vesicles in response to an action potential.

In addition to these binding properties for small ligands, Synaptotagmin interacts with SNARE proteins, SNARE complexes, the clathrin adaptor protein AP-2, as well as with lipid membranes (Li et al., 1995; Zhang et al., 1994). Many of these interactions have been shown to be modulated by Ca^{2+}. In considering the function of Synaptotagmin, it is clear that these interactions could be the means by which Synaptotagmin regulates the fusion step during transmitter release, the ability of vesicles to translocate to and dock at the membrane, or the rate of endocytosis. To resolve the significance of these myriad interactions in vivo genetic analysis of synaptotagmin (syt) mutants was undertaken, first in the fly (Broadie et al., 1994; DiAntonio and Schwarz, 1994; DiAntonio et al., 1993b; Littleton et al., 1993, 1994) and Caenorhabditis elegans (Jorgensen et al., 1995; Nonet et al., 1993) and subsequently in the mouse (Geppert et al., 1994).
B. SYNAPTOTAGMIN PHENOTYPES AT THE NMJ

Null mutations of syt, also called syt1 (see in a later section), are poorly viable and first were believed to be incapable of surviving past the first instar larval stage (DiAntonio et al., 1993b). However, by segregating homozygotes away from their more robust heterozygous siblings and by raising them under appropriate conditions, it is possible for them to survive to the third instar and even to early adulthood (Loewen et al., 2001). Therefore, data are now available for the phenotypes of syt null alleles for embryonic, first instar and third instar NMJs, as well as from weaker alleles. The electrophysiological phenotype of null alleles is severe—approximately a 10- to 40-fold reduction in the amplitude of the evoked response—at each of these stages (Broadie et al., 1994; Loewen et al., 2001; Okamoto et al., 2005; Robinson et al., 2002; Yoshihara and Littleton, 2002). Yet, as expected from the persistent viability of the flies, transmission is never abolished. The remaining evoked responses and other physiological parameters have therefore been examined to determine precisely what is lacking in these mutants.

One hypothesis has been that Syt serves as a fusion clamp that prevents the SNARE proteins from interacting in resting Ca\(^{2+}\), but which releases the SNAREs and thereby permits vesicle fusion when Ca\(^{2+}\) is bound (Littleton et al., 1994). Some suggestion of this mechanism might be taken from the observation that mini frequency is modestly increased at both embryonic and third instar NMJs that lack Syt (Broadie et al., 1994; DiAntonio and Schwarz, 1994; Littleton et al., 1994; Loewen et al., 2001). In this model, the removal of Syt would cause the constitutive fusion of synaptic vesicles as soon as they dock at the active zone, and the evoked response would be reduced because of the inability to accumulate readily releasable vesicles. This model, however, was rejected because the number of minis released was not adequate to explain the drastic reduction in evoked release (DiAntonio and Schwarz, 1994). This finding is also supported by studies in which Synaptotagmin was acutely inactivated (Marek and Davis, 2002). For these experiments, the wild-type Syt was replaced with a syt transgene that included a C-terminus binding site for a fluorescent ligand. After application of the ligand, laser illumination produces reactive oxygen species that rapidly destroy the tagged protein (FlAsH-FALI). In this protocol, evoked transmission could be suppressed within seconds without a concomitant increase in spontaneous release. Thus, Syt plays a positive role by promoting fusion rather than by acting as a fusion clamp to prevent spontaneous release.

The dominant hypothesis is that Ca\(^{2+}\) binding to Synaptotagmin is the necessary and sufficient event to trigger transmitter release in response to an action potential. However, strong evidence for this hypothesis was not easy to achieve. The first obstacle has been the persistence of Ca\(^{2+}\)-dependent transmitter release even in null alleles, a phenomenon also observed in C. elegans and
mice (Geppert et al., 1994; Nonet et al., 1993). Thus, Synaptotagmin can enhance transmitter release, but is not absolutely necessary. In the absence of a complete blockade of transmission, two interpretations were available. Synaptotagmin might not be the Ca\(^{2+}\)-trigger but might instead be a facilitator of release that increases the availability of vesicles for fusion or the probability of their fusing. Alternatively, Synaptotagmin might be a trigger for the EJP, but coexists with a second Ca\(^{2+}\)-sensor that mediates the residual release. The sequencing of the *Drosophila* genome revealed additional synaptotagmin genes that might represent this residual Ca\(^{2+}\) sensor, although none has yet been demonstrated to play this role (see in a later section). Therefore, attempts to clarify the importance of Synaptotagmin have centered on examining the properties of exocytosis in wild-type and *syt* mutants, in the hope of finding an indication that the residual release is using a Ca\(^{2+}\)-sensor that is distinct from that in wild-type synapses. The nonsynaptotagmin Ca\(^{2+}\)-sensor might have a different affinity for Ca\(^{2+}\) or involve the binding of a different number of Ca\(^{2+}\) ions. The latter would be reflected in a different slope for the plot of log[Ca\(^{2+}\)] versus log response amplitude (see in an earlier section). Particularly, if there were two sensors, both of which needed to be activated by Ca\(^{2+}\), the removal of one of them should decrease the slope of this relationship. Recordings from null embryos, however, failed to see such a shift (Broadie et al., 1994). Although some alleles were reported to have a shift in the slope or affinity (Littleton et al., 1994), most recordings indicated a predominant effect on the number of vesicles secreted (V\(_{\text{max}}\)) rather than on the slope or affinity. A change in the number of vesicles secreted could be caused by changes in many aspects of transmission other than a change in the Ca\(^{2+}\) sensor. These studies are exceptionally difficult because the evoked responses are so small in the mutants and because it is necessary to work in very low Ca\(^{2+}\) to determine the cooperativity of the relationship. The most thorough study of evoked release in null *syt* embryos (Okamoto et al., 2005) found a substantial decrease in the slope, consistent with the hypothesis that Syt is the major Ca\(^{2+}\) sensor for exocytosis and that the residual release is driven by a sensor with distinct properties.

Reports of recordings from *syt* alleles sometimes noted that the time course of release was altered, with more release occurring slower or asynchronously than in wild type (Loewen et al., 2001; Yoshihara and Littleton, 2002). This has given rise to speculation that the terminals contain a fast sensor (Syt) and a slow sensor (unknown) governing the residual release. However, a loss of synchrony in transmitter release can be explained by changes in docking, priming, and fusion, in addition to changes in the Ca\(^{2+}\) sensor, and others have observed low levels of fast synchronous release to persist in these mutants (Okamoto et al., 2005).

Some of the most compelling evidence that Synaptotagmin is the major Ca\(^{2+}\)-sensor for triggering fusion has come from studies of the Ca\(^{2+}\)-binding sites in the C2 domain (Mackler et al., 2002; Okamoto et al., 2005). If Synaptotagmin
is the crucial Ca\(^{2+}\) sensor, these studies reasoned, mutation of the aspartate residues that form the Ca\(^{2+}\)-binding site should cause drastic and measurable changes in the Ca\(^{2+}\)-dependent properties of transmission. The Ca\(^{2+}\)-binding site in the C2 domain had been described in detail from structural studies, and the significance of each aspartate for the Ca\(^{2+}\)-dependent properties of Synaptotagmin had been defined for mammalian Synaptotagmin I (Là et al., 1995; Rizo and Sudhof, 1998; Shao et al., 1996, 1998; Zhang et al., 1998b). These mammalian studies implicated the first of the two C2 domains (C2A) as the more important. However, when *syt* null *Drosophila* mutants were supplied with *syt* transgenes carrying mutations that completely disrupted this site (and therewith the ability of the C2A domain to undergo Ca\(^{2+}\)-dependent binding to Syntaxin or phospholipids) synaptic transmission was restored in a manner completely comparable to rescue by a wild-type transgene. Even the slope of the Ca\(^{2+}\)/

response relationship was comparable to that of wild type, indicating that Ca\(^{2+}\) binding by the C2A domain cannot be required for or have a significant influence on the release of transmitter (Robinson et al., 2002). Mutations in the C2B domain resulted in dramatically different consequences (Mackler et al., 2002). Neutralizing the equivalent aspartates in this second C2 domain were sufficient to render the protein inactive in rescue experiments, and expression of this transgene had a dominant-negative effect by suppressing synaptic transmission. Similar dramatic effect was observed in *syt* alleles that either remove the C2B domain or carry a point mutation near the C2B Ca\(^{2+}\)-binding domain (DiAntonio and Schwarz, 1994; Okamoto et al., 2005; Yoshihara and Littleton, 2002).

Together, the evidence from studies of Ca\(^{2+}\)-dependency in the *syt* null and the efficacy of alterations in the Ca\(^{2+}\)-coordinating aspartates of the C2B domain, build a strong case for Synaptotagmin being the crucial sensor for triggering exocytosis. It remains to be determined why some release persists and also why the neutralization of charges in the C2A domain (highly conserved through evolution and partaking in many Ca\(^{2+}\)-dependent interactions) appears to be of so little consequence to synaptic physiology.

C. OTHER FUNCTIONS OF SYNAPTOTAGMIN

The significance of Synaptotagmin for the Ca\(^{2+}\)-dependence of transmitter release does not preclude additional functions of the protein. Electron micrographic studies of central synapses as well as the NMJ (Loewen et al., 2006; Reist et al., 1998) indicate that *syt* mutant terminals have fewer vesicles, although those vesicles present continue to cluster in the vicinity of the active zone. This phenotype suggests a defect in either the biogenesis of synaptic vesicles or in their retrieval from the membrane after fusion. A similar paucity of vesicles at the terminals of *C. elegans* mutants led to the hypothesis that Synaptotagmin
regulates endocytosis (Jorgensen et al., 1995). Such a function is likely to be mediated by the ability of Synaptotagmin to bind the clathrin adaptor protein AP-2 and thereby recruit clathrin to vesicles (Zhang et al., 1994). Quantitative measurements of endocytotic rates have been undertaken in syt mutants by means of a GFP-based reporter of vesicle fusion (Poskanzer et al., 2003). Because endocytosis is coupled to exocytosis, it had not previously been possible to distinguish any direct effect of Synaptotagmin on the former from its clear effects on the latter. Particularly, by using FlAsH-FALI (see in an earlier section) to inactivate Synaptotagmin after a round of exocytosis, it was possible to measure these rates independently. Loss of Syt slows the rate of endocytosis and particularly limits the ability of the synapse to increase its rate of endocytosis in response to increased exocytosis (Poskanzer et al., 2003).

The interactions of Synaptotagmin with phospholipid membranes and with the proteins of the plasma membrane also led to the hypothesis that Synaptotagmin may promote the docking of vesicles at active zones, akin perhaps to the function of C2 domains in various enzymes. Electron microscopy of syt mutants revealed a selective decrease in the pool of vesicles immediately adjacent to the active zone (Reist et al., 1998). Loss of the pool that presumably correspond to vesicles primed and ready for fusion may be a major contributor to the syt phenotype. Mutations of the C2B domain that retain the rest of the protein do not cause these ultrastructural changes (Loewen et al., 2006). This may hold a significant clue for understanding the function of the rest of the molecule.

D. MULTIPLE SYNAPTOTAGMINs IN THE FLY

The Drosophila genome predicts six additional isoforms of Synaptotagmin, but there is little functional data at present concerning any of them (Adolfsen and Littleton, 2001). Syt4 is most homologous to mammalian Synaptotagmins 4 and 11 and, like Syt1, is abundant in the nervous system where it can be found enriched at synapses. At the NMJ it is present in muscle cells, appearing in puncta near the synapse (Adolfsen et al., 2004), but it can also be found in neurosecretory endings, including peptidergic terminals at the NMJ (Bohm and Schwarz, unpublished data). Within the CNS it is not known if Syt4 is predominantly pre- or postsynaptic. Syta and Sytβ (with no immediate relative in mammals but closest to Synaptotagmin 12) are also reported to be concentrated in some neurosecretory endings (Adolfsen et al., 2004), whereas Syt7, homolog to mammalian Synaptotagmin 7, is found in many tissues including nerve and muscle, but has not been observed to be concentrated at synapses. The remaining genes, syt12 and syt14 are the least understood, although low levels of Syt14 have been observed by in situ hybridization in the embryonic CNS (Adolfsen et al., 2004).
Of these additional Synaptotagmins, only Syt4 has been analyzed genetically. Null alleles [from the excision of a P-element (Adolfsen et al., 2004), or made by homologous recombination (Bohm and Schwarz, unpublished data)] are homozygous viable, fertile, and overtly normal. In NMJ morphology and EJP amplitude, the third instar syt4 mutants are similarly normal. The analysis of null alleles does not support earlier suggestions that syt4 could account for residual transmitter release in syt1 null larvae. My laboratory had reported that syt4 transgenes could rescue synaptic transmission in syt1 mutants, but this was in error (Robinson et al., 2002), as was an earlier report on overexpression of syt4 that concluded that syt4 regulates transmitter release by interfering with syt1 function (Littleton et al., 1999). However, at the NMJ of third instar larvae, postsynaptic Syt4 translocates to the plasma membrane in response to activity and this may represent the release of an unidentified retrograde signal (Yoshihara et al., 2005). Also, in embryonic synapses at hatching stages, an electrophysiological phenotype has been reported: whereas in wild-type embryos high-frequency trains of stimuli result in an increase in minifrequency, this was not observed in the syt4 nulls. The effect on minis could be restored by the rescue of syt4 expression in the muscle, consistent with a model in which muscle activity releases a retrograde messenger that modulates the release of minis from the presynaptic terminal (Yoshihara et al., 2005).

E. SUMMARY OF SYNAPTOTAGMIN FUNCTION AT THE FLY NMJ

The analysis of syt1 mutations indicates an important role for Synaptotagmin in promoting the fusion of vesicles in response to an action potential. Because syx1 and n-syb mutants are also required for the EJP, Syt and the SNAREs are part of the same release pathway. The function of Syt is likely to be closely tied to its ability to bind Ca\(^{2+}\) ions. The Ca\(^{2+}\)-binding site of the C2A domain, however, appears rather insignificant, whereas the Ca\(^{2+}\)-binding site in the C2B domain has an essential role. The residual release of vesicles in response to an action potential or elevated cytosolic Ca\(^{2+}\) remains a puzzle. If it is due to the presence of a redundant synaptotagmin gene, the gene has not yet been identified. In addition to a role in promoting fusion, Syt is likely to promote vesicle recycling and vesicle docking.

IX. Exocyst at the NMJ

The SNARE complex is not the only protein complex that is implicated in membrane traffic. The genetic screens for secretory defects in yeast that uncovered mutations in SNARE proteins also uncovered a complex that is
frequently referred to as the exocyst (EauClaire and Guo, 2003; Lipschutz and Mostov, 2002). The eight components of this complex are Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 and their phenotypes in yeast include blocking a late stage in vesicle transport to the plasma membrane. In budding yeast, vesicles are transported into the growing bud but fail to fuse at their target sites (Novick et al., 1980). One of the most intriguing aspects of the exocyst is that it localizes to the site of vesicle fusion, that is, at the bud tip when daughter cells are growing and at the bud neck when membrane is being added for cytokinesis (Finger and Novick, 1997; Finger et al., 1998). In contrast, the SNARE proteins are uniformly distributed within the plasma membranes of yeast. The exocyst might therefore be responsible for docking vesicles at target membranes.

With so many other mechanistic parallels between membrane traffic in yeast and at the synapse (Bennett and Scheller, 1993), the question naturally arose as to whether the exocyst plays a similar role in the nervous system, particularly with regard to targeting synaptic vesicles to active zones. The exocyst complex was purified from mammalian brain (Hsu et al., 1996) and antibody studies suggested an important function in membrane traffic in epithelial cells (Hazuka et al., 1999). A murine mutation of sec8 was discovered (Friedrich et al., 1997), but it caused lethality at a very early stage of development and therefore was unsuitable for more detailed studies of membrane traffic.

The analysis of exocyst mutations and neuronal function therefore commenced with the identification of sec5 mutations in Drosophila (Murthy et al., 2003). As previously discussed regarding syx mutations, the analysis is complicated by the fact that members of this complex have vital functions and cell-lethal phenotypes. sec5 null alleles are cell lethal when clones are made in the eye or female germ line (Murthy and Schwarz, 2004; Murthy et al., 2003). However, as in the case of syx, maternally contributed sec5 mRNA is sufficient to permit the embryo to develop and, in the case of sec5, larvae hatch out and can survive for up to 3 days. These larvae, however, do not achieve their expected size but rather remain at the border of first and second instars. This can be attributed to the need for sec5 in constitutive membrane addition. Although there is sufficient maternal Sec5 in homozygous null embryos to support normal development, by hatching it has fallen to 29% of wild-type levels and by 48 h after egg laying (AEL) to 11%, at which point no further growth is observed. By 72 h AEL, Sec5 levels are barely detectable, no more than 3% of wild type. At the NMJ this is manifest in the arrest of muscle growth and a similar arrest in the development of the synapse. At 96 h, motoneurons on muscles 6/7 have bouton counts appropriate for 48 h larvae (Murthy et al., 2003). Therefore, by studying neurons from larvae that were 72 h AEL or older, the significance of Sec5 could be assessed in the almost complete absence of the protein. It was found that these neurons had severe defects in membrane traffic. If neurons were dissociated from larval brains they were incapable of re-extending an axon. If a reporter gene was
turned on at this stage, it could be synthesized but was not efficiently trafficked to the cell surface (Murthy et al., 2003). These defects notwithstanding, the synapse at the NMJ continued to function and the EJP was slightly larger at 96 h than it had been at 48 h, perhaps reflecting the limited formation of new release sites. Although the amplitude of the EJP was well below that of a normal 96 h larva, presumably due to the small size of the NMJ, release per bouton was quite normal. Thus, Sec5 is essential in many membrane trafficking steps, including those that are responsible for the growth of the NMJ and formation of new boutons. However, it is dispensable for the actual targeting and fusion of synaptic vesicles once a bouton has formed.

Mutations have been reported in the exocyst components sec6 (Beronja et al., 2005; Murthy et al., 2005) and sec15 (Mehta et al., 2005). Although these studies have not closely examined synaptic transmission or the NMJ per se, the mutant phenotypes reported are consistent with the conclusion that the exocyst is not needed for the release of neurotransmitter. Synaptic phenotypes at the NMJ have been examined for mutations in sec8 (Liebl et al., 2005) and through the use of RNAi to reduce levels of sec10 (Andrews et al., 2002). In each case the release of transmitter was unaffected. The significance of the exocyst or of individual components of it for synaptic development, however, remains an area of great interest because it has the potential not only to permit but perhaps also to direct the addition of new boutons presynaptically and the expression of receptors postsynaptically.

To a large extent, synaptic transmission can be thought of as a variation on exocytosis in all cells, including yeast, but with additional regulatory components, such as Synaptotagmin, added on. Sec5 and the exocyst as a whole, however, are a counter example. The exocyst is a protein complex that is essential in yeast and in the constitutive trafficking pathway for many, perhaps all, membrane proteins, and yet it is dispensable for transmitter release. One possible explanation is that the specialized architecture of the active zone provides a unique mechanism for the docking of synaptic vesicles at release sites. If so, this mechanism may substitute for the normal role of the exocyst (Murthy et al., 2003).

X. Other Mutations of Proteins on the Target Membrane

A. Rop/UNC-18/N-Sec1

As discussed earlier, neither mutations in SNARE-coding genes nor in genes encoding components of the exocyst have phenotypes that are adequate to explain how synaptic vesicles are preferentially clustered near and docked at the active zone. From studies of mammalian synapses, there are several major proteins that may contribute to these events, but which presently have received
less attention in *Drosophila*. One of these is a Syntaxin-binding protein called Sec1 in yeast and n-Sec1 or Unc-13 in mammals and *C. elegans*, respectively. Mutations in the *Drosophila* homolog gene were recovered fortuitously in a screen for *ras* mutants and were given the name of *rop* (Harrison et al., 1994). The phenotype of *rop* mutants, including developmental abnormalities in embryos, strongly implicated *rop* as necessary for both constitutive and synaptic exocytosis, which was subsequently confirmed by the analysis of point mutations (Wu et al., 1998). Overexpression of Rop, however, inhibited transmitter release (Schulze et al., 1994). These findings have led to conflicting interpretations, according to which Rop is either an inhibitor of transmission or a necessary promoter of vesicle release. The enhancement of synaptic transmission by mutations in the Rop-binding region of Syt appeared to favor a model in which this protein restricts the availability of Syt for SNARE complex formation (Wu et al., 1999). This has proven controversial due to the difficulty of determining *in vivo* the extent to which protein interactions have actually been prevented (Matos et al., 2000; Wu et al., 2001). From mammalian studies, it is clear that the n-Sec1/Unc-18 protein is not bound to Syntaxin when Syntaxin is complexed with the other SNARE proteins. It is therefore most attractive, at present, to envision Rop as a protein that cycles on and off Syntaxin, priming it for activity in transmission, but then releasing it so that Syntaxin could bind to the other SNAREs. Such a cyclical role could explain both its requirement for secretion and the ability of overexpressed Rop to interfere with transmitter release.

B. UNC-13 AND VESICLE PRIMING

Unc-13, a protein that has been extensively studied in both *C. elegans* and the mouse (Rhee et al., 2002; Richmond et al., 1999), may also regulate the release apparatus. The prevailing model, from studies in those organisms, is that Unc-13 can regulate the state of Syntaxin and thereby influence its ability to form SNARE complexes (Richmond et al., 2001). Unc-13 appears to be a crucial focus for modulating the strength of synaptic transmission in response to changes in second messengers, particularly diacylglycerol (Rhee et al., 2002). *Drosophila* lacking *unc-13* have a severe phenotype: a complete loss of both the embryonic EJP and minis (Aravamudan et al., 1999) and an accumulation of docked vesicles. In addition, the levels of Unc-13 may be modulated by second messenger systems (Aravamudan and Broadie, 2003).

C. CAST/ERK AT THE ACTIVE ZONE

The monoclonal antibody nc82 has been a favorite for the analysis of synaptic structures due to its remarkable specificity for active zones. The gene
encoding the nc82 epitope has been cloned and shown to encode the fly homolog of ELKS/CAST (Wagh et al., 2006), a protein whose localization to active zones has also been established in vertebrates (Ohtsuka et al., 2002; Wang et al., 2002). In Drosophila, the locus was given the name bruchpilot (brp). Although the function of ELKS/CAST is not known, it binds to a protein called RIM-1 that in turn binds to Unc-13 (see in an earlier section). Although no mutation in the fly homolog has yet been reported, protein levels were knocked down by means of RNAi expression (Wagh et al., 2006). Reducing nc82 immunoreactivity produced a reduction in the amplitude of the EJP, although minis persisted normally. A striking ultrastructural phenotype confirmed the importance of the protein in organizing the active zone. In Drosophila, many although not all active zones possess an electron dense structure, sometimes called a T-bar. This structure may be akin to the ribbons and similar structures seen at some mammalian synapses. RNAi to Drosophila CAST/ELKS prevented the formation of these T-bars.

**XI. Mutations in Peripheral Synaptic Vesicle Proteins**

**A. SYNAPSIN**

The Synapsin family was one of the first major synaptic proteins to be identified and it associates with the cytoplasmic surface of synaptic vesicles. It was recognized early to be the subject of phosphorylation by both cAMP- and Ca\(^{2+}\)-dependent protein kinases (Greengard et al., 1993). Consequently, there has been much hope and speculation that it might be a crucial control point for such synaptic phenomena as modulation by amine transmitters and paired-pulse facilitation. By and large, these expectations have not been met by the phenotypes of knockout mice (Rosahl et al., 1993, 1995), although the presence of three partially redundant genes in the mouse makes this analysis more complicated. Murine data suggest a role for the Synapsins in regulating the availability of reserve pool vesicles during prolonged neuronal activity (Chi et al., 2001; Sun et al., 2006). In Drosophila, the presence of a single synapsin (syn) gene, albeit an alternatively spliced gene, has assisted its genetic analysis (Godenschwege et al., 2004). The syn mutant phenotype, however, remains astonishingly mild—undetectable at the level of cellular analysis at the NMJ. No alteration was detected in the ultrastructure of these synapses, including the density of synaptic vesicles in the vicinity of active zones. Thus, no indication was found that Synapsins were essential for coupling vesicles to the local cytoskeleton. In addition, the properties of the EJP and minis were unchanged from control. Even with 2 s of 5 Hz stimulation, no differences were observed, with a modest
degree of synaptic depression in both mutant and control. However, the flies have distinct behavioral abnormalities, including learning defects, in both adult and larval stages (Godenschwege et al., 2004; Michels et al., 2005). Thus, the importance of Synapsin to the functioning of the nervous system was confirmed, but the specific cell biological function of this vesicle protein has not been identified at fly synapses. More detailed physiological tests may eventually reveal a subtle electrophysiological phenotype.

B. CYSTEINE STRING PROTEIN AND HSC70

The cysteine string protein (CSP) is another protein associated with the cytoplasmic surface of synaptic vesicles (Chamberlain and Burgoyne, 2000). It has homology to DNAJ/Hsc40 proteins and through its J domain can interact with the chaperone Hsc70. Therefore, studies have focused on a likely role as a chaperone protein that can assist in protein folding and refolding (Braun and Scheller, 1995; Braun et al., 1996). Consistent with such a model, Hsc70 mutants have also been shown to have a phenotype at the fly NMJ. The significance of such a protein on synaptic vesicles remains unclear. It may involve interactions with Syntaxin and Synaptotagmin (Bronk et al., 2001, 2005).

The phenotype of Csp mutants in Drosophila has been crucial to determining its physiological significance. CSP cannot be central to the process of transmitter release and exocytosis—transmitter release persists in null mutants (Zinsmaier et al., 1994). Nevertheless, synaptic function is not normal. Particularly at elevated temperatures synaptic function is impaired causing smaller evoked responses (Dawson-Scully et al., 2000; Ranjan et al., 1998; Umbach et al., 1994). This reduction does not appear to be due to a decrease in Ca\(^{2+}\)-channel activity. The synaptic defects are accompanied by a progressive degeneration of neurons and eventual paralysis and death. The importance of the interaction with Hsc70 was confirmed by the isolation of mutations in that gene and the finding that they give rise to phenotypes very similar to those of Csp (Bronk et al., 2001), although some effects of Csp may be independent of Hsc70 (Arnold et al., 2004; Bronk et al., 2005). The prevailing model, at present, is that repeated cycling of synaptic vesicles, particularly at elevated temperatures, results in the production of denatured proteins. It is not yet known who the critically vulnerable proteins are. CSP on the vesicles, working with Hsc70 and perhaps, an additional associated protein (Tobaben et al., 2001), can restore proper folding of these proteins and prevent the ensuing degeneration and loss of synaptic strength (Fernandez-Chacon et al., 2004). In this regard, the neurodegenerative phenotype of CSP may become an interesting model for the study of degenerative disorders.
Nearly two decades of work on the release of transmitter at the *Drosophila* NMJ has produced a sizeable collection of mutations and a great deal of phenotypic analysis. Mutations have been generated in all the central components of the release machinery and in many additional regulatory and peripheral components of the nerve terminal. It is interesting that mutant screens in *Drosophila* have not yet identified essential elements of the exocytosis apparatus that were not previously identified by biochemical means. However, by bringing electrophysiological and ultrastructural analysis *in vivo* to the study of synaptic components, the system has made substantial contributions to our understanding of synaptic function in all organisms.

The task of elucidating synaptic mechanisms is not over. One of the most significant unanswered questions centers on the active zone. The prominent electron density of this structure suggests numerous highly specialized components, but few have as yet been identified or subject to genetic analysis. We still do not understand why synaptic vesicles accumulate in terminals and cluster near active zones. Nor do we understand how synaptic vesicles are selectively targeted to these sites rather than elsewhere on the cell surface, while other cargo-carrying vesicles do not fuse at the active zone. Similarly, the mechanistic relationship of minis to EJPs remains unclear. Perhaps the most important question for synaptic function entails understanding the differences between synapses. Why does one synapse has a high probability of release while another has a low probability? How is the release of peptidergic granules for modulatory and neuroendocrine function distinct from the release of small clear vesicles? How are synapses specialized for tonic or phasic release of transmitter? It is likely that the *Drosophila* NMJ will continue to figure prominently in the exploration of these issues.

**References**


diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. \textit{Cell} \textbf{108}, 121–133.


