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Cellular Traffic: A Synaptic Detour

RICHA RIKHY and K S KRISHNAN*
Department of Biological Sciences, Tata Institute of Fundamental Research
Homi Bhabha Road, Colaba, Mumbai- 400005

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Membrane limited vesicles sustain the intracellular traffic of proteins and lipids in the cell. Soluble proteins are moved around mainly as luminal contents while membrane proteins and most lipids are transported as part of the vesicle membrane. The components are removed by vesicle fission and delivered to appropriate sites by vesicle fusion. Endocytosis and exocytosis are defined in the context of nutrient uptake and secretion. This broad area of membrane traffic has seen unprecedented growth in the past ten years. Understanding molecular mechanisms governing membrane traffic, in addition to garnering insights in to a most fundamental aspect of biology, has implications for development and neuronal function. It is also likely that it will provide important leads to understanding a wide variety of human disorders. Enhanced imaging techniques, new and powerful methods in microscopy; some like evanescent wave microscopy, capacitance fluctuation measurements and ultra-structural analyses, seemingly made to order for vesicle studies, have altered the ways in which we view the workings of the cell and its organelles. Genetic and molecular studies have had a profound, perhaps the most impact. This review attempts to highlight some of the developments and emerging unity of thoughts in the basic processes of membrane fusion and fission. The bias towards the synapse and our particular focus on genetic studies in *Drosophila* is not by design but due to our inability to encompass all that is known and all that has been suggested in other studies in a minimalist view. And of course, the hope that some times the detour has more scenic spots and the traffic flows easier.

Key Words: Synapse, Membrane traffic, Exocytosis, Vesicle recycling, Endocytosis

Introduction

Discrete organelles, cellular components, and the cytoplasm itself is membrane limited and exchange of large molecules is effected by fusion and fission. One aspect of biology that is not often highlighted is that all living organisms arose in an unbroken chain of fusion and fission events from the primordial "First Cell". The processes of fusion and fission are fundamental to living, allowing no gross errors. The relative disposition of proteins and other membrane components is retained while surface and concentration changes are minimized in transfer of macromolecules by vesicle fusion and fission. For the cell, the plasma membrane serves as a barrier for outside material and prevents loss of cellular contents. Endocytosis is the means of choice for nutrient uptake, signal transduction, receptor down-regulation, membrane retrieval, vesicle recycling and maintenance of cell polarity. Secretion and release of signaling molecules is by exocytic fusion. Within a typical eukaryotic cell there are other arrays of discrete cell membranes, which are in continuous metabolic exchange with each other. Different proteins reside in organelles such as the endoplasmic reticulum, the Golgi complex and the mitochondrion. These proteins are translocated to the cytoplasm and proteins synthesized in the cytoplasm likewise get translocated into organelles, are modified and trafficked further. Figure 1 illustrates the organelles that are locales of fusion and fission at some stage in their lifetime. A highly regulated and specialized form of trafficking occurs at the terminals of neurons.

The mechanistic details of these two primal cellular processes remain rather poorly understood. The problem is seen in better perspective by comparing the fusion event to a hypothetical situation of having to fuse two water filled balloons to a seamless single unit without spilling a molecule of water or fission to pinching off a small water filled

*Corresponding address: *Email:* ksk@tifr.res.in

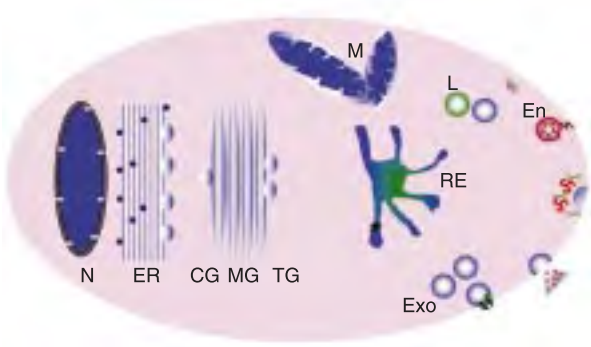

Figure 1. Cellular sites of Fusion and Fission. In a typical cell, vesicles with soluble proteins in their lumen and lipids and membrane proteins integral to their surface are budded off from different organelles. These in turn fuse to other organelles and deliver their cargo. Some of the examples depicted are endoplasmic reticulum (ER) to the cis Golgi (CG). These events occur between the cis, medial and trans Golgi (CG, MG, TG) compartments followed by transport or even exocytosis (Exo) of the contents of the vesicle to the outside of the cell. Compensatory membrane retrieval by endocytosis (En) can occur by multiple pathways. Vesicles endocytosed from the plasma membrane may be processed by fusion with lysosomes (L) or the recycling endosome (RE). Mitochondrial (M) and nuclear (N) division and propagation during the cell cycle is also subject to fusion and fission events.

balloon from a larger one. This is certainly not the same as pinching off a tad of dough from a large bolus. Even while physiology with ion transport has reached such fine heights of analysis as to be able to describe precisely the movement of ions, their gating and inactivation mechanisms (Jiang et al. 2003a, b), the descriptions of similarly mechanical processes of fusion and fission remain incomplete. Here we make an attempt to provide a minimalist and intuitive description of the molecular mechanisms of fusion and fission events in synaptic transmission. We have tried our best to give important data and thoughts their due but are aware that some areas, results and models are dealt with much less a detail than they deserve.

The Synaptic Vesicle Cycle

Literature tends to classify exocytosis and endocytosis as constitutive and regulated to distinguish secretion related to cellular metabolism from activity or signal related release. In regulated form of exocytosis as it happens at the neuronal synapse the ligands are kept ready for release in small membrane limited vesicles and are released by fusion of the vesicle membrane to the plasma membrane upon arrival of an action potential. The synaptic vesicle membranes are in turn retrieved by endocytic fission. This 'Vesicle recycling' is responsible for keeping the synapse ready for the next impulse (figure 2). We believe the synapse is an ideal model for understanding broad questions in trafficking, particularly those related to regulated forms of exocytosis and endocytosis.

While membrane events involved in fusion and fission are very similar and share many common features in all forms of traffic in the cell, this review will be restricted to discussing the present state of knowledge in exocytic release of neurotransmitters and endocytic retrieval of synaptic vesicles. In this review we bring together information revealed through several, biochemical, genetic, physiological and cell biological studies on membrane trafficking and recycling at the synapse. We especially focus in detail on the specialized mechanisms of this 'Synaptic Vesicle Cycle' and facts and ideas that have emerged from *in vivo* genetic studies.

Questions and Broad Objectives

The major outstanding questions in exocytosis and endocytosis, we believe, are quite similar and they are as follows.

Where are the Sites of These Events?

Do these occur on all membrane sites with equal facility or are they restricted to specific supramolecular assemblies on bilayer membranes in cells? What are the different forms of these basic

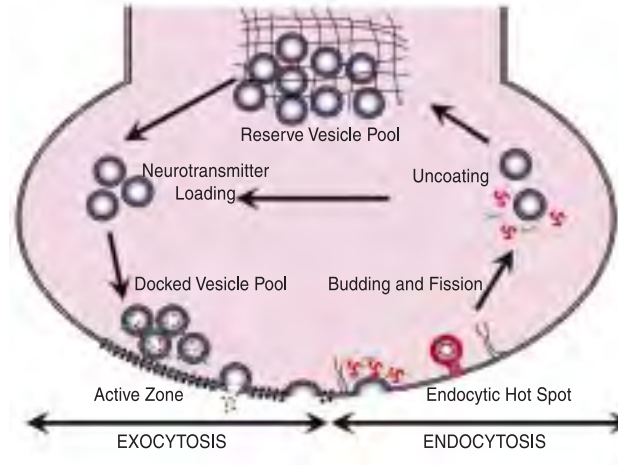

Figure 2. The Synaptic Vesicle Cycle. At the synapse vesicles packed with neurotransmitter are docked at the active zone. The reserve pool of vesicles is centrally located and serves to replenish the docked pool. Exocytic fusion results in release of the neurotransmitter. The vesicles are retrieved and recycled at the peri-active zone (Endocytic hot spots) by Clathrin mediated endocytosis. The series of events involved in exocytosis and endocytosis comprise the synaptic vesicle cycle.

fusion and fission processes and how are they related to their purported immediate end.

What Machinery in Each Case is Involved?

Is all the machinery broadly similar or are there different mechanisms at play in different cellular processes? How in different processes the end result of seamless new units is achieved without loss of specific components and contents? This is important because the balance of ratios of surface area to volume will involve addition or removal of membrane components if not dramatic alteration in membrane properties after the events.

What Triggers such Events?

And what mechanisms translate such a trigger to mobilizing the machinery? Do the triggers differ and how are the distinct machineries selected? A significant part of our understanding will come from considerations of regulation and the energetics of these processes.

In What Order and How?

How are the various elements in the molecular catenae that effect endocytosis and exocytosis ordered? What really are the dynamics of molecular interactions that result in the re-arrangement of proteins and lipids during fusion and fission? It is also critically important to know the relative time scales and links between fission and fusion events.

These are by no means all the questions but the broad aspects that need to be clarified in initiating the synthesis of a mechanistic view of these processes and will have implications to our understanding of the biological events mediated by such processes.

Methods used in the Study of Exocytosis and Endocytosis

It is pertinent to discuss some methods of studying exocytosis and endocytosis at this point as it would make the discussion of literature that follows easier.

Electrophysiology

The very initial details of synaptic transmission were obtained by Katz and colleagues at the frog neuromuscular junction (NMJ) (Katz 1962). They showed that "transmitter is liberated from nerve endings in packets of fixed size", by recording spontaneous miniature synaptic currents at the NMJ. This concept enshrined in the quantal theory of synaptic release further led to the vesicle hypothesis when chemical synapses were seen to contain uniform membrane limited structures by electron microscopy. Electrophysiological recordings carried out at the synapse measure the release of neurotransmitter in evoked and spontaneous conditions. An Excitatory post-synaptic potential, results from the depolarization of the post synaptic membrane caused by an excitatory transmitter. This is a product of the quantal size that is usually directly proportional to the actual size of the vesicles and quantal content which represents the number of quanta released in response to a pre-synaptic action potential. The release under conditions of low calcium could be modeled as a Poisson's distribution and the quantal content estimated from frequency of failure in responses to stimuli. Such measurements are now used routinely in neurons from different organisms in both the peripheral and central nervous system. Electrophysiology is still the most important single method of studying synaptic function. The first electrical correlates of synaptic release were seen by Fatt and Katz (Fatt & Katz 1952). It is remarkable that Katz and colleagues soon arrived at virtually all the details we broadly understand about synaptic release with simple electrical measurements (del Castillo & Katz 1954, Katz & Miledi 1965), in an era where even chemical basis of neurotransmission particularly in the central nervous system was being questioned by the likes of Eccles. It was only after intracellular recording from spinal motorneurons by Coombs, Eccles and Fatt that revealed depolarizing excitatory and hyper-polarizing inhibitory postsynaptic potentials, that Eccles abandoned the electrical hypothesis for synaptic transmission (Coombs et al. 1955).

In Vitro Methods

Synaptic vesicles can be isolated in a relatively pure form due to their uniformity and hydrodynamic properties. In fact synaptic vesicles are perhaps the only sub-cellular organelles other than mitochondria and nuclei that can be effectively purified. Early purification attempts were made from tissues containing mostly a single type of vesicle. These preparations were then injected into rabbits or mice and antibodies obtained that often stained the nervous systems in rats (Whittaker et al. 1972, Buckley & Kelly 1985, Matthew et al. 1981). These antibodies were used in pull down assays to

identify proteins and obtain the sequences of genes coding for them. Synaptobrevin and Synaptotagmin were identified in this manner (Matthew et al. 1981, Trimble et al. 1988).

In vitro trafficking assays have been used to identify molecules involved in vesicle trafficking through the Golgi complex. In one such ingenious assay, membrane trafficking was monitored by following the transport of vesicular stomatitis virus encoded glycoprotein. Donor membranes infected with the virus were prepared from CHO cells that lacked the enzyme N-Acetylglucosaminyl Transferase. Acceptor membranes possessed this enzyme. The two membranes were mixed and incubated at 37^0C in the presence of Tritiated UDP-GlcNAc. The radiolabel would be incorporated into VSV-G only if membrane trafficking was accurate. This assay showed that trafficking required cytosol, ATP and was sensitive to N-ethyl maleimide and lead to the discovery of N-ethyl maleimide sensitive fusion factor (NSF) (Balch et al. 1984a).

Lipids dispersed in aqueous media under appropriate conditions form a closed bi-layer structure called liposome. These are competent to fuse with each other in a manner membrane limited structures are, and will in fact fuse with cells under appropriate conditions. Recently it has been shown, that the endocytic protein Dynamin tubulates lipid dispersions and this property of Dynamin is shared by a few other endocytic proteins like Amphiphysin and Endophilin. Liposomes loaded with proteins synthesized in *vitro* and lipid dispersions tubulating in presence of endocytic proteins have been useful in study of biochemical activities in fusion and fission. They have allowed an assay for the fusion properties of various SNARE proteins and the tubulation and pinching ability of Dynamin in fission. Recent biochemical studies in presence of lipid tubules of mutant forms of Dynamin have helped delineate the function of sub-molecular domains in Dynamin earlier indicated by behavioral and genetic studies (Grant et al. 1998).

In Vitro Reconstitution and Structural Studies

The advent of protein engineering has allowed an assay for the activity of proteins *in vitro*. This in addition with membrane fractions or artificially synthesized lipid membranes has led to the identification of mechanistic aspects of fusion and fission. There are several *in vitro* mixing experiments; in one such assay v and t SNARES were shown to be sufficient for membrane fusion of distinct vesicles. The v-SNARE vesicles contained a quenched mixture of two fluorescent phospholipids. These fluorescent "donor" vesicles were allowed to fuse with "acceptor" vesicles that contained only unlabelled lipids. Upon fusion concentrations of both donor and acceptor are reduced in the mixed bilayer resulting in a decrease in quenching and a consequent increase in donor fluorescence (Weber et al. 1998).

In addition to the above, X ray crystallography has established the binding specificity of SNARES with each other. It is now clear that SNARES are highly twisted and coiled coil proteins which bind in a parallel orientation. Each bundle is made of 4 α helices, one from a v-SNARE and the others from t-SNAREs along with conserved leucine zipper like motifs are found in the center of the fusion complex. The surface of the complex contains distinct hydrophobic, hydrophilic and charged regions which have been hypothesized to be essential for binding of regulatory factors during membrane fusion (Sutton et al. 1998).

Electron Microscopy (EM)

Early EM studies identified electron dense filamentous regions called 'active zones' where vesicles were docked and possibly released by exocytosis. Heuser's freeze-fracture EM studies showed the exocytic process in unprecedented detail (Heuser et al. 1971). Electron microscopic analyses of neurons after HRP uptake initially led to the vesicle recycling hypothesis and Ikeda used EM methods to first suggest that the *shibire* mutant may be blocked in endocytosis (Heuser & Reese 1973, Kosaka & Ikeda 1983). These results led to the identity of Dynamin as a major molecular player in endocytosis when the DNA sequence coding for the *shibire* locus was seen to be highly homologous to the earlier identified motor protein. Detailed structural information on Dynamin coated tubules is currently obtained from electron micrographs. In an impressive use of the tubulation assay done on EM grids, it was shown that the pitch of the Dynamin oligomer ring changes during the transition from GTP bound form to GDP bound form, fuelling a model of physical displacement of the budding vesicle from the plasma membrane surface (Stowell et al. 1999).

Time-resolved Capacitance Measurement

Both endocytosis and exocytosis are expected to change the net surface area and this in turn may cause transient capacitance changes. Patch clamp measurements are ideally suited for measuring such changes in membrane capacitance. With minimal modifications of existing patch clamp set ups an analysis for capacitance fluctuations have been done (Neher & Marty 1982). This technique has been exploited fruitfully in systems such as the neuroendocrine cells, peptide secreting nerve terminals and chromaffin cells (Leszczyszyn et al. 1990, Wightman et al. 1991). Measurements are usually done either with a whole cell patch or in a perforated patch configuration. These measurements also allow the simultaneous measurement of calcium currents which trigger exocytosis. This allows high temporal resolution and has been useful in providing an estimate of the number of granules in the releasable pool of vesicles and obtaining information about processes that affect the sizes of the vesicles.

Amperometry

Amperometry relies on the detection of compounds released at the synapse with suitable detectors (Chow et al. 1995). The technique has been used in studies on chromaffin cells which release adrenalin. The release is detected by oxidation or reduction of the secreted product on the surface of the carbon fiber electrode placed close to the cell. The sensitivity of Amperometric methods allows detection of secretion from individual cells. This has been used to characterize temporal and spatial relationships between calcium influx into a neuron and subsequent fusion events in isolated chromaffin cells. These techniques typically gather information near the electrode tip and therefore are useful in gaining spatial information especially about hot spots of secretion.

Optical Imaging of Fluorescent Dyes and Fluorescently Labeled Proteins

Lichtman and colleagues first described activity dependent release and uptake of fluorescent dyes at the snake motor terminals (Lichtman et al. 1985). The development of styryl dyes such as FM1-43 that stain a wide variety of preparations in an activity dependent fashion greatly enhanced the sensitivity and convenience of use of imaging methods (Betz et al. 1992a, Betz & Bewick 1992b). The styryl dyes are amphipathic and partition to the membrane and are also easily washed off. There are many modified styryl dyes available and they have distinctly different fluorescence properties and on and off rates with respect to distribution across the membrane cytosol interface. All of the dyes have large increases in quantum yield when partitioned into membranes thus enhancing their sensitivity. They have been especially significant in identifying vesicle pools at the synapse.

Advances in optical microscopy like confocal microscopy, fluorescence microscopy and near-field scanning optical microscopy, have now made it possible to locate and observe the dynamics of molecules in cellular environments in "real" time. This has been possible with the achievement of high resolution, sensitivity and selectivity, as well as the ability to image in three dimensions. In laser fluorescence microscopy (LFM), a laser excites tag molecules of high fluorescence quantum yields that have been used to label specific proteins or lipids. With the appropriate implementation, it is possible to analyze molecular interactions by use of FRET, FCS and polarization measurements in such systems (Varma & Mayor 1998). To achieve good three-dimensional resolution, LFM is implemented confocally. Confocal imaging of GFP tagged proteins and especially *p*H sensitive GFP have been useful in following the events of the synaptic vesicle cycle (Lippincott-Schwartz & Patterson 2003).

Two-photon Microscopy and Multi Photon Excitation

The laser intensities in LFM are high and hence fluorophores can photobleach rapidly and other cellular components that absorb energy at the laser wavelength could be damaged. The drawbacks associated with LFM can largely be avoided through two-photon excitation of fluorescence. Two-photon excitation is based on the ability of fluorophores to be excited by the simultaneous absorption of two photons each with half the energy. These allow for better resolution and resilience to photo-bleaching. Recently, Multi-Photon Fluorescence Microscopy has emerged as a new optical imaging technique. In this type of microscopy excitation is confined to the optical section being observed. Illuminating light of a wavelength approximately twice that of the

absorption peak of the fluorophores being used, is employed. So, for example, if a fluorescein isothiocyanate (FITC) labeled sample is being observed, excitation in a Two-photon system can be achieved at approximately 1000nm (FITC has an absorption peak at around 500nm). This means that, essentially, excitation of the fluorophore will not be achieved at this wavelength, thus eliminating photobleaching in the bulk of the sample (Denk et al. 1990, Maiti et al. 1997).

Evanescent Wave Microscopy

Total internal reflection fluorescence microscopy allows selective illumination of a portion of the cell very near to the cover slip to which it is attached. When a beam of light is incident on a surface, at an angle greater than a critical angle that depends on the refractive index of the two interfaces, it is totally reflected. Interference between the incident and reflected beams results in a narrow electro-magnetic field, the evanescent wave, which is parallel to the optical surface. The energy of the field falls exponentially as the distance from the interface. This has a width of the order of tens of nanometers and has a wavelength equal to that of the incident light. This phenomenon has been used effectively to image events of single vesicles budding from the membrane by employing GFP tagged proteins (Merrifield et al. 2002). Use of GFP tagged neuropeptide and GFP Actin in evanescent wave microscopy has allowed the visualization of vesicle docking sites at the plasma membrane and highlighted the significance of Actin in promoting fission (Zenisek et al. 2000, Blanpied et al. 2002). Such studies provided direct confirmation of "hot spots" of endocytosis that have been proposed earlier (Estes et al. 1996).

Perturbational Methods

Methods that specifically alter properties of individual macromolecules have led to the delineation of their roles in exocytic and endocytic mechanisms. Many pharmacological agents, drugs and toxins fall in this category. Notable are Botulinum, cholera toxin and the black widow spider venom. For example; N-ethyl maleimide has a specific effect on NSF, tetanus toxin cleaves Synaptobrevin and different botulinum toxins cleave Synaptobrevin, Syntaxin or SNAP-25 (Rossetto et al. 2001). Injection of antibodies and peptides into the neuromuscular junction of large synapses such as the lamprey giant synapse has helped relate molecular properties known from biochemical assays to in *vivo* function. Injection or transfection of dominant negative constructs has been widely used especially for perturbation of protein function by injecting protein domains.

In addition there are various compounds which can be used to specifically perturb intracellular calcium. These include caged calcium compounds, which result in release of calcium in a stimulus dependent manner. There are other treatments, which result in exocytic release in the absence of a calcium stimulus such as hypertonic sucrose shock and black widow spider venom. These have been of importance in estimating the docked pool of vesicles.

Genetic manipulations of the kind available in yeast, *Drosophila*, *C. elegans* and mouse have contributed significantly to relating in *vivo* functions to the biochemistry of identified synaptic molecules. The availability of unique reagents, complete genome sequence and facility of manipulation bestows an unprecedented advantage on *Drosophila* studies. The resultant behavioral phenotype, particularly those that are conditional, and cellular and physiological assays of mutants have helped in elaborating the mechanisms of vesicle cycle (Stimson & Ramaswami 1999). It is possible not only to study the mutational phenotype in individual cells in real time but it is even possible to localize these defects to sub-cellular structures (Guha et al. 2003, Sriram et al. 2003).

Mechanisms of Exocytic Release

Sub-cellular Location of Exocytosis at the Synapse

Having a defined site for a process ensures spatial organization, and this is a key to order and efficiency. Specialized exocytic regions are created by close apposition of the pre-synaptic and the post-synaptic membranes especially at sites where synaptic vesicles are present. These so called 'active zones' are sites at which neurotransmitters are released. Electron microscopic studies showed that the active zone is characterized by a regular electron dense structure that is adjacent to the pre-synaptic membrane and has filamentous material that originates and extends deep into the synaptic cytosol (Heuser et al. 1971). EM tomography has provided excellent views of docked vesicles

positioned in 30 nm dense protein scaffold whose composition remains to be elucidated (Harlow et al. 2001). At other synapses freeze etch EM views provide evidence that 100 nm Spectrin like proteins connect vesicles to the active zone (Hirokawa et al. 1989). Molecular studies have further identified several proteins that localize to the active zones. Two of these large proteins Piccolo and Bassoon (~400 KDa) have Zinc finger motifs and are associated with the active zone cytomatrix (Zhai et al. 2001, Shapira et al. 2003). RIM1 (180KDa) and Munc13 (200KDa) are the others known to be present (Refer to table 1 for a summary on some known proteins at the active zone). It is presumed that these proteins endow the active zone with scaffolding functions which are essential for fast neurotransmitter release. Evanescent wave microscopy in gold fish has shown that exocytosis occurs repeatedly at the same preferred sites presumably the active zones. However some release though not regular was observed at sites other than active zones (Zenisek et al. 2000).

Exocytosis

The basic mechanism of exocytosis involves the recognition of the target membrane by the vesicle and their fusion to release the contents. This fusion is heterotypic in that it is between two distinct membranes. This occurs in a signal dependent manner to release the contents of the vesicle onto the post synaptic cell which can be another neuron or a muscle. The triggering signal is calcium provided by its entry through voltage-gated calcium channels activated by a depolarizing wave in the axonal membrane (Katz 1962).

N-ethyl maleimide was found to disrupt in *vitro* trafficking of VSV-G and this led to the discovery of N-ethyl maleimide sensitive fusion factor or NSF (Balch et al. 1984a, b, Braell et al. 1984, Malhotra et al. 1988, Weidman et al. 1989). Soluble NSF attachment proteins called SNAPs were identified as proteins that bind NSF and have obligatory role in its function (Clary & Rothman 1990a, Clary et al. 1990b). Syntaxin was later found as the neuron specific membrane receptor for NSF and α-SNAP (Bennett et al. 1992). In addition Myc tagged NSF α-SNAP was used to obtain a set of SNARES as pull down products. The eluted proteins after micro-sequencing were found to be Syntaxin1, VAMP2 and SNAP25. These proteins together form a 20S complex and this gives fusion competence at different stages of the secretory pathway. The current models based on binding experiments combined with Fluorescence Resonance Energy Transfer (FRET) and crystal structure analysis of Syntaxin, SNAP25 and VAMP suggest that VAMP, Syntaxin and SNAP25 interact to form a coiled coil structure (Lin & Scheller 1997, Sutton et al. 1998). The binding orientation of the three proteins is parallel to each other and four α helices, two from SNAP25, one each from VAMP and Syntaxin are held together. This brings the vesicle and the target membrane in close apposition and is sufficient to mediate fusion.

The proposal that a 20S complex formed by VAMP, Syntaxin, SNAP25, α SNAP and NSF mediating fusion was one of the first mechanistic models for exocytosis (Beckers et al. 1989). The SNARE hypothesis accounts for many features of fusion, particularly target recognition, in different cellular processes. The v-SNAREs on the vesicle (VAMP) and the t-SNARES on target membrane (SNAP25 and syntaxin) bind each other and mediate fusion (Schiavo et al.1995). NSF was characterized as an ATPase and α SNAP is necessary for loading NSF onto the SNARE complex made up of VAMP, syntaxin and SNAP25 (Sollner et al. 1993a,b). It was thought that the energy needed to dissociate the 20S complex was provided by the ATP hydrolysis and this mediated fusion (Barnard et al. 1997). However many *in vivo* experiments carried out suggest that NSF is needed at a step referred to as priming, before actual vesicle fusion.

Yeast experiments were conducted to determine the step of the fusion process at which SNAP and NSF function was essential. In a set of experiments marked by their relative simplicity and elegance, yeast homotypic vacuolar fusion was assayed using vacuolar membrane vesicles prepared from two different strains one that contained proPho8p, a pro-alkaline phosphatase and the other that contained the Pep4p, which is a protease that converts the former enzyme into its active form. When such vesicles did fuse it was possible to assay for alkaline phosphatase activity as a measure of fusion competence. It was determined that it was sufficient to allow NSF and SNAP activity prior to actual mixing of the two membrane fractions containing different SNARES for fusion to occur. This led to the hypothesis that ATP

Table 1. *Proteins Involved in Exocytosis*

Protein Name	Functional category	Subcellular Location	Assigned Function
SNARE COMPLEX			Fusion
v-SNARE vesicle associated membrane protein (VAMP)/ synaptobrevin	Transmembrane CC domain	Vesicle	SNARE complex formation in fusion
t-SNARE SNAP25	Transmembrane CC domain	Plasma membrane at the active zone	SNARE complex formation in fusion, interaction with calcium channels
Syntaxin	Transmembrane CC domain	Plasma membrane at the active zone	SNARE complex formation in fusion, interaction with calcium channel and synaptotagmin
α, β, γ SNAP		Cytosol	Loads NSF to the SNARE complex
NSF (N ethyl maleimide sensitive fusion factor)	AAA ATPase	Cytosol	Disengages v-SNARES to interact with t-SNAREs
CALCIUM REGULATION			Stimulate or modulate exocytosis or fusion
Calmodulin	Binds Ca^{2+}	Cytosol	Ca Buffering Hypomorphic mutants have a decreased evoked response
Frequenin	4 EF hands for Ca binding	Cytosol	Activates Guanylyl cyclase and may function overexpression shows increased release in Drosophila
Synaptotagmin	Transmembrane Phospholipid and calcium binding C2 domains	Vesicle membrane	Binds Syntaxin and interacts with calcium channels. Is the putative calcium sensor at the synapse
Protein Kinase C	C2 domain binds lipids and Ca	Cytosol	Phosphorylation of proteins at the nerve terminal
Cysteine String Protein	String of 11 cysteines present	Vesicle membrane	Modulates calcium channel release at the synapse
Complexin	15 KDa proteins	Cytosol	Binds SNARE complex core domains and stimulates Ca dependent neurotransmitter release
ACTIVE ZONE			Scaffolding
Piccolo	400kDa protein Zn finger motifs	Cytomatrix at the active zone	Scaffolding and assembly of the active zone
Bassoon	400kDa protein Zn finger motifs	Cytomatrix at the active zone	Scaffolding and assembly of the active zone
RIM1	180kDa protein	Cytomatrix at the active zone	Priming of vesicles
Munc13	200kDa protein	Cytomatrix at the active zone	Enhances the rate of priming and refilling of the readily releasable pool of vesicles
RAB CYCLE			
Rab3	Small GTPase	Cytosol	Recruiting vesicle, fusion regulation

Table 1 ***(contd.)***

Protein Name	Functional category	Subcellular Location	Assigned Function
Rabphilin	C2 domain	Shuttles between cytosol and vesicle membrane	Recruitment of vesicles to the active zone
Noc2	Homology to Rabphilin	Cytosol	Phosphorabphilin has reduced affinity for membranes Binds to synaptic vesicles.
Tomosyn			Binds Rab3a and regulate exocytosis
EXOCYST COMPLEX			
Sec3p			Polarized cell growth: neurite outgrowth; vesicle tethering ?
Sec4p		Vesicle membrane	
Sec5p			Anchor vesicles to the exocyst complex
Sec6p			
Sec8p	PDZ domain	Golgi complex	Important for delivery from Golgi to plasma membrane. Binds PSD95.
Sec10p	CC domain		Hormone secretion and not synaptic transmission
Sec15p			Binds vesicle exocyst protein Sec4p
Exo70p			
Exo84p			
SM FAMILY			Responsible for regulation of rates of fusion
nSec1/ Munc18	Conserved domains to many â sheet proteins	Cytosol	Binds syntaxin and acts at a step prior to Munc13
Munc13	Mentioned in section on active zone	Cytosol	Modulation of exocytosis
Doc2	Double C2 domain protein	Vesicle membrane	Calcium signal responsive modulation of fusion
Mint1/X11L	Phosphotyrosine and PDZ domains transmembrane	Plasma membrane	Binds Neurexins and is responsible for maintaining synaptic polarity
Neurexin	Transmembrane	Plasma membrane	Presynaptic receptor for a latrotoxin

hydrolysis by NSF was a part of the priming step prior to vesicle fusion and probably for the step of resolution of SNARE complexes formed in previous events (Nichols et al. 1997, Mayer et al. 1996). The *Drosophila* mutant *comatos*e shows temperature sensitive paralytic behavior (Siddiqi & Benzer 1976). This is perhaps the only instance of a neural phenotype for an NSF mutant. Although *Drosophila* has another gene coding for NSF, the *comatose* mutant phenotype implicates NSF in synaptic vesicle cycle (Kawasaki et al. 1998, Pallanck et al. 1995). This has been used in double mutant combinations with the Na channel mutant in elegant behavioral and electrophysiological experiments demonstrating that in spite of the *comatose* block there are some vesicles that are fusion competent and are possibly depleted only on multiple rounds of synaptic vesicle recycling (Sanyal et al. 1999, 2001).

In the squid giant axon it was found that on injection of tetanus toxin, which cleaves VAMP, the number of vesicles increased with a decrease in evoked response. This suggests that VAMP is needed for fusion and not for docking (Hunt et al. 1994). *Drosophila* has also been useful in assaying functions of many members of the SNARE complex. There are *Drosophila* mutants in VAMP (n-Syb), SNAP25 and Syntaxin in addition to NSF (Kidokoro 2003). In experiments expressing tetanus toxin in fly nervous system, in n-Syb null mutants as well as in Syntaxin mutants it was shown that nerve stimulation did not evoke synaptic currents at the embryonic neuromuscular junction. However there was an increase in number of morphologically docked vesicles in both these mutants. Spontaneous release was however present in VAMP mutants and absent or decreased in Syntaxin mutants. These data clearly suggest that there is an absolute need for Syntaxin in mediating fusion (Deitcher et al. 1998, Sweeney et al. 1995, DiAntonio et al. 1993a, b, Broadie et al. 1995, Schulze et al. 1995). SNAP25 temperature sensitive mutants show a decrease in evoked response without a change in spontaneous nerve transmission at restrictive temperatures. A decrease in action potential induced release in neurotransmitter has been observed in the case of rats injected with Botulinum toxin, which cleaves SNAP25 at the 9th amino acid (Schiavo et al. 1992, Poulain et al. 1993). This has been shown to decrease the calcium sensitivity of the synapse to neurotransmitter release. In the case of *Drosophila* mutants at permissive temperatures the evoked release is twice that of the control animals and calcium sensitivity of the synapse is increased (Rao et al. 2001). Hypomorphic n-Syb and Syntaxin mutants also reduce the calcium co-operativity of neurotransmitter release. This suggests that SNARE complex has a role in calcium sensing or is closely linked to the calcium sensor, Synaptotagmin.

There are several different v and t SNAREs at different organellar compartments (ER, Golgi and plasma membrane) of the secretory pathway. SNARE hypothesis implied in addition that SNARES defined the membrane interactions at different stages of the secretory pathway (Rothman & Warren 1994). Specificity is inherently needed for accurate targeting to the cognate membranes. However the 7S SNARE complex has been found to be stable with different members of the Syntaxin, SNAP25 and VAMP family (Weber et al. 1998, Fasshauer et al. 1999, Yang et al. 1999). Further a plasma membrane SNARE can be substituted with an endosomal SNARE and the stability is not altered significantly (Bhattacharya et al. 2002). The summary for SNARE function is a part of table 1 in the section for fusion. Specificity is currently thought to be imparted by distinct or combinations of several proteins such as the Rabs. The Rab cycle is discussed subsequently.

Calcium Sensor for Exocytosis

The entry of calcium triggers the vesicle fusion event. Since the discovery that calcium serves as a signal for exocytosis there has been a search for molecular targets that serve to sense the increase in calcium at the synapse on the arrival of a stimulus. Proteins such as Synaptotagmin have been thought to be calcium sensors at the synaptic terminal. There are several other proteins which play a role in modulating the calcium current by their interaction with the voltage gated calcium channels and they are also highlighted in this section.

One of the monoclonal antibodies raised against synaptic membranes pulled out a 65KDa protein (Matthew et al. 1981). This protein, Synaptotagmin (p65) was further sequenced and found to have homology with the regulatory domain of Protein Kinase C (C2) (Perin et al. 1990, Wendland et al. 1991, Shao et al. 1996). It is a single pass synaptic vesicle membrane protein, which has two cytoplasmic C2 domains towards its C terminus C2A and C2B which are responsible for phospholipid and calcium binding (Perin et al. 1991, Petrenko et al. 1991, Brose et al. 1992) (Refer to table 1 for other proteins which have calcium binding domains). Thirteen isoforms of Synaptotagmin have been identified at the last count (Craxton 2001). Three calcium ions bind to C2A and two to C2B in Synaptotagmin1 (Umbach et al. 1998, Sutton et al. 1990, 1999). However the intrinsic affinity of Synaptotagmin for calcium is low and it is thought that the binding of the SNARE complex proteins may regulate this affinity.

Several lines of evidence suggest that Synaptotagmin has a post-docking role in exocytosis. The physiological function of Synaptotagmin was first deduced by injection of inhibitory peptides and antibodies. Injection of antibodies to the C2A domain of Synaptotagmin or antimorphic peptides

inhibits fast exocytosis in the squid giant axon and results in accumulation of vesicles (Elferink et al. 1993, Bommert et al. 1993). This suggested a role for Synaptotagmin at a step past docking. The Synaptotagmin null mouse dies early in development and functional studies in cultured hippocampal neurons suggest that calcium evoked postsynaptic current is decreased whereas there is no significant alteration in the amplitude of the response as compared to wild type mice during release by hypertonic shock (Geppert et al. 1994b). These defects however can also arise if Synaptotagmin has a role in keeping the vesicles close to zones of calcium increase on the arrival of an action potential.

The SNARE complex proteins as well as Synaptotagmin interact with voltage gated calcium channels and this can potentially provide a mechanism for calcium regulation (Leveque et al. 1994, Martin-Moutot et al. 1996, Takahashi et al. 1996). The SNARE complex proteins and voltage gated calcium channels colocalise at the active zone in the frog neuromuscular junction. Modulation of calcium currents through the voltage gated calcium channel has been studied by co-expression of different proteins in xenopus oocytes followed by calcium current recordings. Such experiments have revealed that Syntaxin, SNAP25, Synaptotagmin and the calcium channel are capable of interacting with each other (Sheng et al. 1996, Sheng et al. 1997, Zhang et al. 2002b, Zhong et al. 2002). The inward current properties are normal in xenopus oocytes with all the proteins co-expressed (Charvin et al. 1997, Wiser et al. 1997, Tobi et al. 1998, Shao et al. 1997). In addition the C2 domain of Synaptotagmin binds Syntaxin in a calcium dependent manner. Thus there is an effective mechanism at the synapse for coupling calcium entry with as little a time lag as possible to SNARE complex formation and exocytosis of primed vesicles.

Several studies in *Drosophila* and *C. elegans* seeking to elucidate the role of Synaptotagmin as a calcium sensor *in vivo* have resorted to the mutational approach (Kidokoro 2003). *Drosophila* hypomorphic mutants in Synaptotagmin show a defect in calcium evoked nerve transmission (Reist et al. 1998, Littleton et al. 1993a, β Littleton et al. 1994, Littleton et al. 1999, Desai et al. 2000, Yoshihara & Littleton 2002, Marek & Davis 2002, Robinsonet al. 2002). Calcium dependency of neuro-transmission is lost in these mutants, results that are in accord with the role of Synaptotagmin as a calcium sensor. Other studies propose a role for Synaptotagmin in endocytosis or synaptic vesicle recycling and as an inhibitor of fusion. The mini frequency of *Drosophila* larvae in null mutants is higher than wild type and this suggests a role as a negative regulator of fusion. This does not hold true in the studies carried out in mice. The *C. elegans* Synaptotagmin mutant has no defect in synaptic transmission and survives normally to adulthood but shows a defect in synaptic vesicle recycling (Jorgenson et al. 1995). The C2B domain of Synaptotagmin binds to AP2, which is a part of the endocytic machinery, and the implications of this finding are discussed in the section on synaptic vesicle recycling (Jorgenson et al. 1995, Li et al. 1995).

In addition to the above experiments there are several biochemical and biophysical studies which elucidate the structure and affinity of Synaptotagmin for calcium in the presence of membrane phospholipids. These suggest that the C2 domains of Synaptotagmin interact strongly with anionic lipids such as phosphatidyl serine and this interaction is enhanced with calcium binding (Davis et al. 1999). Further, there has been a debate as to whether Synaptotagmin will bind membranes in cis (vesicle membrane) or trans (plasma membrane). It is known that the C2B domain has affinity for binding phosphatidyl inositol 4, 5 bisphosphate. A calcium dependent increase in binding is thought to result in penetration through the membrane and subsequent increase in proxi-mity between the vesicle and target membrane (Sugita et al. 1996, Schiavo et al. 1996). Finally a current model for the action of Synaptotagmin unequivocally suggests that it can bring about major conformational changes in a calcium evoked manner leading to and mandatory for fusion (Fernandez et al. 2001).

Another protein known to regulate the SNARE complex formation in a calcium dependent manner is called complexin. Complexins are small proteins highly enriched in the brain at synapses. Molecular, biochemical and structural studies suggest that complexins bind and stabilize the SNARE complex core domains and increase the probability for SNARE mediated vesicle fusion (reviewed in Marz & Hanson 2002).

There are other proteins, which perform a regulatory role preceding calcium induced exocytosis. Cysteine string protein (CSP) was found to bind to calcium channels and regulate its function (Magga et al. 2000). CSP was first identified as a protein present in the nerve terminals of *Drosophila* (Zinsmaier et al. 1990) and later shown to be specific to synaptic vesicles. It was found to modulate the function of calcium channels in oocytes (Gundersen & Umbach 1992, Leveque et al. 1998). *Drosophila* mutants in CSP show a temperature sensitive paralytic behavior and a loss of evoked release of neurotransmitter whereas the spontaneous release remains unaltered (Umbach et al. 1994). A favored hypothesis for CSP function implicates it in coupling the entry of calcium into the synaptic terminal with neurotransmitter release. A cysteine rich domain mediates a cooperative interaction with hsp70, a molecular chaperone thought to be important for the stabilizing protein complexes (Braun et al. 1996). Mammalian CSP has been shown to potentiate the activity of HSC70 and this led to the suggestion of a role in removal of a Clathrin coat during endocytosis (Chamberlain et al. 1997). However *Drosophila* CSP mutants do not show any defect in FM1-43 tracer dye uptake at restrictive temperatures and this argues for a preferential role in calcium modulation during neurotransmitter release as against vesicle recycling (Ranjan et al. 1998). In yet another instance the J domain of CSP has been shown to interact with G proteins in an ATP dependent manner resulting in the inhibition of N type calcium channels suggesting that CSP may play an essential role in fine tuning neurotransmitter release (Magga et al. 2000). Table 1 is a list of several molecules known to be involved in exocytic fusion and its regulation.

Exocyst Complex

Constancy of quantal content is achieved by maintaining a fraction of available vesicles in a ready to release state. It is also necessary that the transmitters are released close to where receptors in post synaptic cells are found in abundance. Thus synapses have evolved elaborate mechanisms where by active zones of release are juxtaposed to receptor clusters. Readily released vesicles need a molecular station to dock and release their contents when the signal arrives. Such docking centers would be expected to both recognize vesicles and provide the machinery for fusion. Part of this machinery is likely to be conserved in all secretory processes while others may be synapse specific. The identification of an 'exocyst' complex emerged from a genetic dissection of the yeast secretory pathway. This complex of proteins is not necessary for formation of vesicles or for intracellular trafficking but is specifically distributed at sites of membrane fusion consistent with a role in vesicle tethering. It was found to assemble at the tip of the growing yeast bud. It consists of 10 proteins of which Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p are associated with one another (TerBush et al. 1996). Sec3p is responsible for attaching to the membrane and the Sec15p is important for tethering to vesicles (Salminen et al. 1989, Guo et al. 1999). Several small GTPases are attached to different membranes and through their interactions regulate exocytic and endocytic processes. Sec4p/Rab, Rho and Ral are essential for transport and recruitment of vesicles to zones of vesicle docking and exocytosis (Guo et al. 1999, Moskalenko et al. 2002, Brymora et al. 2001, Sugihara et al. 2002, Guo et al. 2001, Zhang et al. 2001).

This complex was elucidated in mammals by similarity of several of the proteins to their yeast counterparts (Guo et al. 1997, Kee et al. 1997, Matern et al. 2001). The complex assembles at exocytic zones in neurons, neuronal growth cones and in epithelial cells on the apical membrane where polarized delivery of vesicles to sites of cell surface expansion, is necessary. The whole complex can be pulled down with microtubules. It is present at the microtubule organizing center in undifferen-tiated neurons and is recruited to the tips of growing neurons during differentiation and growth. Here it colocalises with synaptic vesicle markers such as Synaptotagmin. When the neurite outgrowth is inhibited, vesicles accumulate in the cytosol (Hazuka et al. 1999, Vega et al. 2001).

The *Drosophila* Sec5p mutants show defects in neurite outgrowth and neuromuscular junction formation. However, there is no electrophysiological defect in synaptic transmission (Murthy et al. 2003). This has also been observed in the developing rat brain where the expression of the exocyst complex precedes differentiation and

expression of functional synaptic markers. These studies strongly suggest that the exocyst complex is necessary for synaptic growth but not for synapse function. On the contrary an RNAi approach knocking out Sec10 has no assayable neural phenotypes in *Drosophila* other than in ring gland endocrine function (Andrews et al. 2002). These experiments lead us to conclude that the exocyst complex is not likely to be required for synapse function in mature neurons.Refer to table 1 for a summary of the proteins which form the exocyst complex.

Vesicle Priming and Docking

Many proteins have been directly implicated in a step which is distinctly different from actual fusion and this has been called vesicle priming. This step prepares vesicles to respond to increases in calcium concentration. There are some proteins which regulate the inappropriate fusion of proteins that belong to the SNARE complex. SNARE complex proteins are essential for bringing about fusion of two membranes but they certainly do not function in docking, targeting or recruitment of vesicles to the cognate membranes. The Sec1/Munc (now popularly referred to as the SM family) family of proteins performs this function (Toonen & Verhage 2003) and some members of this family with known functions are a part of table 1.

Nsec1 (neuronal sec1) binds with nanomolar affinity to the syntaxin at the plasma membrane. This complex formation is mutually exclusive to that of the Syntaxin-Synaptobrevin- SNAP25 complex (SNARE complex) *in vitro* (Pevsner et al. 1994a, b, Yang et al. 2000). The Nsec1 orthologue in *Drosophila* is the product of a gene called *Rop* and over expression of this protein results in a decrease in neurotransmitter release (Schulze et al. 1994). Null alleles and point mutations in members of Sec1 family lead to a block in vesicle fusion (Schekman 1992, Verhage et al. 2000). These results indicate that Nsec1 has a general regulatory role in synaptic vesicle exocytosis. Nsec1 has also been shown to interact with Rab GTPases. A three dimensional crystal structure of the Syntaxin Nsec1 complex indicates that it facilitates the interaction of Rab proteins with effectors which might release Syntaxin to form the SNARE complex (Misura et al. 2000). Rab effector binding to Nsec1 releases it from Syntaxin in yeast (Webb et al. 1997). The *C. elegans* Unc18 is homologous to and performs the same function as Nsec1 (Sassa et al. 1999). Sec1 is also important as a trafficking protein between specific target membranes since it is a Rab effector and Rab binding is found to be important for exit of Syntaxin from the Golgi complex (Rowe et al. 1999). SM proteins interact with other proteins such as Doc2 and Mint and this may contribute to some of their functions. Mints may be proteins important for maintaining cell polarity (Rongo et al. 1998). Mint proteins interact with Neurexins which are the pre-synaptic receptors for latrotoxin (Biederer & Sudhof 2000). Doc2 is enriched on synaptic vesicles and contains C2 domains like Synaptotagmin (Verhage et al. 1997, Orita et al. 1995). This implies a role in phospholipid and calcium binding during evoked neurotransmitter release. Injection of inhibitory peptides against Doc2 results in inhibition of evoked release. However Doc2 null mutant mice have a mild phenotype and so the function of Doc2 is debatable (Sakaguchi et al. 1999, Mochida et al. 1998).

As mentioned above Munc13 dissociates the Nsec1-Syntaxin complex. Munc13 binds the same N terminal region of Syntaxin as used by Nsec1 and in *C. elegans* this interaction is thought to displace Nsec1 (Sassa et al. 1999). It also binds to Nsec1 and Doc2. Munc13 contains a diacylglycerol phorbol ester binding C1 domain and was isolated in a classical screen done in *C elegans* for uncoordinated movement (Maruyana & Brenner 1991, Lackner et al. 1999, Miller et al. 1999). Munc13 enhances the rates of priming and refilling of the readily releasable pool of vesicles. In mice, *C. elegans* and *Drosophila* mutants for Munc13, there is a decrease of evoked junction potentials (Aravamudan et al. 1999, Augustin et al. 1999, Richmond et al. 1999). The readily releasable pool of vesicles is strongly reduced but the number of docked vesicles remains the same which suggests a role for this protein in the vesicle priming event or the conversion of vesicles from the docked state to the fusion competent state. Unc13 has been shown to change the confirmation of Syntaxin to an open one so that it is ready for fusion (Richmond et al. 2001, Voets et al. 2001). This suggests that Munc13 acts after the Munc18 step.

In figure 3 A we represent exocytic fusion as conceived in several models. This leaves the actual fusion process itself some what unclear and in fact many non-bi-layer structures for lipids during

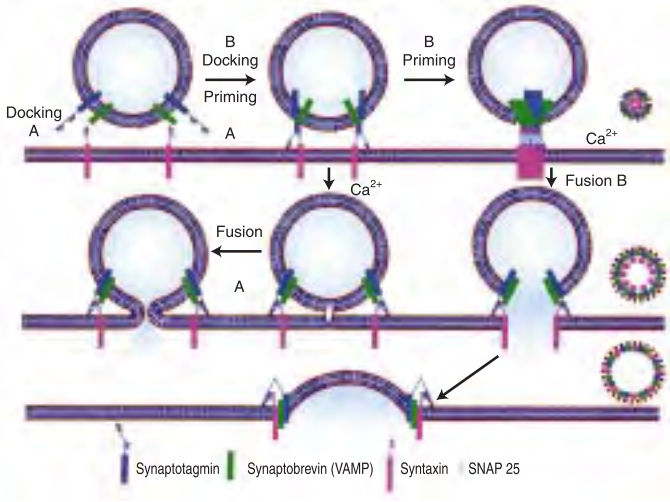

Figure 3. Mechanism of SNARE mediated Membrane fusion. The SNARE complex is responsible for vesicle fusion. Model A represents current notions. Model B represents modifications that account for features of fusion event not fully explained in Model A. In Model A, while docking is mediated by SNARE proteins, the v-SNARES are disengaged from each other at the priming step by NSF mediated resolution of the SNARE complex enhancing t-SNARE/v-SNARE interaction and close apposition of vesicles with target membrane. The entry of calcium triggers fusion of the vesicle membrane with the target membrane. The exact mechanism of fusion is left to be imagined and it does not clarify the maintenance of geometry and vectoriality of integral membrane proteins in the vesicles. In the modified form (Model B) vesicles dock via SNARES in a step similar to Model A. Priming results in resolution of the SNARE complex machinery by NSF and facilitation of 'fusion pore forming complex'. Calcium entry triggers the opening of the fusion pore and conformational changes in pore complex leading to collapse of vesicles on to target membrane.

exocytosis have been proposed to account for the transition to vesicles attached to vesicles integrated to target membrane. In figure 3B we illustrate a view where by protein-protein interaction and lateral mobility consistent fusion pores could be formed and expanded. Some of the proteins discussed above could line such fusion pores. It is equally conceivable that there are specific proteins devoted to such expandable pores. This idea is not implausible as gap junctions do transiently form pores and respond to calcium signaling. The important implication for such a model is that it achieves seamless fusion without recourse to extraordinary structures but within the confines of protein structural changes and diffusion on membrane surfaces.

Rab Cycle

Rabs are small GTPases, which are thought to mediate directional vesicle trafficking. It has already been mentioned above that Nsec1 interacts with Rab proteins. All Rabs cycle between an active GTP bound form and an inactive GDP bound form. Rab proteins are associated in their GTP bound form to membranes of the resident organelle and upon membrane traffic GTP is hydrolyzed and the Rabs dissociate from the membrane. This has been confirmed at the synapse where Rabs dissociate from synaptic vesicles upon exocytosis. Rab3a is a negative regulator of exocytosis. Rab3a mutants in mice and *C. elegans* survive and have mild phenotypes but detailed analysis suggests that the neurotransmitter release indeed is enhanced two fold. However, spontaneous release as well as quantal size is not affected (Geppart et al. 1994a, Geppart et al. 1997, Geppart & Sudhof 1998).

Rab3a binds to other proteins such as Rabphilin, Noc2 and Rab interacting protein (RIM) in a GTP dependent manner. Rabphilin3a is regulated by phosphorylation events and binds to the synaptic vesicles (Foletti et al. 2001). It contains C2 domains capable of interacting with calcium and phospholipids. Phosphorabphilin has reduced affinity for membranes. Rab3a mutant mice display mislocalisation and reduced abundance of Rabphilin (Geppert et al. 1994a). However, Rabphilin repression and over-expression experiments indicate that it is a positive regulator of exocytosis (Chung et al. 1995). The Rabphilin mutants in *C. elegans* also genetically interact with VAMP independent of Rab3a (Staunton et al. 2001). These results indicate a direct potentiation of the SNARE complex by Rabphilin.

RIM is a modular protein located at the active zone and may play an important role in localizing synaptic vesicles. It binds to Munc13, Synaptotagmin and Liprins and forms a pre-synaptic scaffold at the active zone. RIM mutants in *C. elegans* are viable and have a normal level of docked vesicles. The number of fusion competent vesicles is reduced but the calcium competence was not affected (Koushika et al. 2001). The open form of Syntaxin suppressed these defects. All these observations suggest that RIM function is important for priming and regulating the normal probability of release. The results mentioned above imply that Rab3 function is important at a late step in exocytosis of the synaptic vesicle cycle. From the above discussion it is clear that the molecular players of the

Rab cycle machinery have not been assigned accurate functions but we summarise some of the known details as a part of table 1.

Mechanisms of Endocytic Retrieval at the Synapse

Cells internalise extracytoplasmic materials, nutrients and components of its surface by a variety of mechanisms. Phagocytosis, pinocytosis, Clathrin mediated endocytosis, Caveolin mediated endocytosis and endocytosis independent of Caveolin and Clathrin have been distinguished in different contexts. These differ drastically in the sizes of membrane retrieved and the functions they have been implicated in (reviewed in Conner & Schmid 2003). The primary event of vesicle recycling at the synapse is an endocytic retrieval of the components of synaptic vesicles from the presynaptic plasma membrane. Much less is known about the molecular mechanisms of vesicle recycling at the synapse as compared to exocytic release of neurotransmitters. When the synapse is stimulated at a high frequency it gets depressed or fatigued and takes several seconds to recover. It was observed that the number of quanta released far exceeded the number present at the nerve terminal at any time (Bittner & Kennedy 1970). This indicates that the vesicle complement can be limiting if not replenished. Synaptic exocytosis is possibly one of the fastest cellular events given that repeated release can happen within a millisecond interval. Endocytic mecha-nisms seem to take place in time scales of seconds. The replenishment of molecules at the synapse through de novo synthesis is slow and occurs by transport from the cell body. The cell body however may be in some cases placed meters away from the synaptic terminal, where all the communication takes place. Thus local recycling is essential for maintenance of sustained synaptic release even in normal conditions. Endocytosis is not only responsible for obvious membrane retrieval but also for recycling of proteins important for various steps of exocytosis. John Heuser and Bruno Ceccarelli independently articulated this problem and provided the first evidence in the seventies (Heuser & Reese 1973, Ceccarelli et al. 1973). Here we discuss the existing literature on the sites of retrieval, signals triggering endocytosis and molecular mecha-nisms mediating invagination of membrane and fission of vesicles in the recycling process. Refer to table 2 for a summary on the proteins which form a part of the endocytosis machinery.

Types of Synaptic Vesicle Recycling

Vesicle recycling at the synapse can occur by at least two different pathways. These pathways are known to exist in different locations and have remarkably different kinetics. The slow pathway is by Clathrin mediated endocytosis and occurs at distinct sites away from the active zone and a fast pathway involves recycling at the active zone where the entire content of the vesicle is not released and therefore is popularly referred to as 'kiss and run' recycling.

Coupling of endocytic and exocytic processes at the synapse was first ascertained in 1970s by studies at the frog neuromuscular junction. Heuser and Reese used EM analysis to observe the frog neuromuscular junction after treatment with horseradish peroxidase (Heuser & Reese 1973). The samples were fixed and analysed after high stimulation and they found invaginating vesicles and vesicles containing HRP at distinct sites away from the active zone. Ceccarelli and co workers found that when the same neuromuscular junction is stimulated at lower rates of exocytosis vesicle internalization occurs at sites close to the active T-junctions where exocytosis is prominent (Ceccarelli et al. 1973). The Heuser and Reese model has been more popular as it matches the receptor mediated endocytosis mechanism found in non-neuronal cells. On the other hand the endocytic intermediates seen at the active zone which bolster the Ceccarelli model could really have been artifacts of exocytosis. Nevertheless the two models suggest spatially distinct pathways for synaptic vesicle recycling: the active zone pathway, which operates when low frequency stimulus is given and the non-active zone pathway which is found on extensive stimulation of the neuromuscular junction. However there is a fair amount of evidence suggesting the existence of both the pathways at the synapse. These different pathways have been observed frequently and in different organisms. We discuss some of the evidence in the next paragraphs.

Temperature-sensitive paralytic mutants in *Drosophila* have been useful in identifying steps of synaptic transmission. *Shibire* flies were found to have a defect in a large GTPase thought to be essential for synaptic vesicle recycling (function is described in the section on Fission) (Kosaka & Ikeda 1983, Koenig & Ikeda 1989, van der Bliek & Meyerowitz 1991).

Table 2. *Proteins involved in Endocytosis*

	Functional category	Subcellular Location	Assigned Function
BUDDING and ADAPTOR PROTEIN COMPLEX			Forms the vesicle coat
α adaptin	Ear and trunk domain	Endocytic hot spots	Part of the adaptor complex AP2, binds to clathrin
β adaptin	Ear and trunk domain	Coat	Part of the Adator complex AP2
μadaptin	YXXψ binding domain	Coat	Part of the adaptor complex AP2 binds to synaptotagmin
AP180	Clathrin binding domain	Coat	Recruits clathrin
Clathrin	Heavy and light chains	Coat protein	Forms the coat which buds a vesicle
Epsin	EH domain, NPF and DPW motifs	Rim of coated pit	Capable of membrane curvature
Eps15	EH domain and DPW motif	Rim of coated pit	Fidelity of synaptic vesicle recycling
Stoned A	NPF motif	Cytosol?	Role in synaptic vesicle recycling
Stoned B	Homology to μ adaptin	Endocytic hot spots	Role in synaptic vesicle recycling
FISSION			
Dynamin	GTPase domain, GED, PH, PRD	Present in Endocytic hot spots	Promotes fission of vesicle
Endophilin	LPAAT domain and SH3 domain	Recruited to endocytic hot spots	Modulates curvature of membrane
Amphiphysin	CC, PRD, SH3	Present post synaptically in Drosophila	Binds to dynamin and synaptojanin
Intersectin	SH3, EH	Present at Endocytic spots	Interacts with WASP, cdc42, dynamin, MAP Kinase and SNAP25
Syndapin	FCH, SH3	Sub synaptic reticulum	Interacts with dynamin and WASP
Grb	SH3	Coat?	Interacts with signaling machinery and dynamin
UNCOATING			
Synaptojanin	Phosphoinositide phosphatase domain, Sac1	Cytosol and vesicle coat	Regulates vesicle uncoating and binds amphiphysin, Endophilin, dynamin and AP2
Hsc70	ATPase	Cytosol and vesicle coat	Uncoating of clathrin coats
Auxilin	DNA J domain	Cytosol and vesicle coat	Targets Hsc70 to clathrin coats
REGULATORS			
Protein kinase C	C2 domains	Cytosol	Phosphorylation of dynamin at the resting nerve terminals

There was no defect in exocytosis in these flies and only recycling was blocked and this is the cause of paralysis at the non-permissive temperatures. Electron micro-scopic sections of retinula synapses in *shibire* flies first depleted at 29^0C and then restored to permissive temperatures showed the presence of long tubular invaginations from almost 100% of the active zones in the first minute (Koenig & Ikeda 1996). Only at later time points is there an accumulation of branched tubules referred to as recycling intermediates seen away from the active zone. Again these temporally and spatially distinct pathways have been suggestive of two distinct mechanisms of synaptic vesicle recycling at the synapse. In flies deficient for Endophilin, a lipid modifying enzyme which is present at the synapse,

it was expected that the Clathrin-mediated endocytosis pathway may be blocked. In one set of studies it was found that the synapse can be sustained at high frequencies due to vesicles at the active zone and it was suggested that these recycle by a Dynamin-independent mechanism, presumably by kiss and run recycling. Endophilin null mutants were shown however, in other studies to shut off vesicle recycling completely in a use dependent manner (Vertstreken et al. 2002, Rikhy et al. 2002).

Capacitance measurement in gold fish neurons could be fitted to a double exponential suggesting that two different pathways of separable time scales operate in recycling (von Gersgorff & Mathews 1997). For the faster mechanism it was found that the pre-synaptic capacitance increases rapidly on the application of a stimulus and then drops immediately with a time constant of almost two seconds. Also two phases of release of catecholamine was seen in adrenal chromaffin cells by Amperometry and capacitance analysis (Wightman et al. 1991). This included a fast and short spike usually followed by a large spike of release. The fast spike was most likely due to transient fusion pores, which do not release all the neurotransmitter present in vesicles. This mechanism allows high speed and efficiency for synaptic vesicle recycling.

The kinetics of synaptic vesicle recycling has been inferred from tracer uptake studies such as HRP or FM1-43. Using these dyes at the frog neuromuscular junction and on hippocampal synapses the internalization time has been found to be 30-60 sec and the total recycling time has been inferred to be 45-75 sec (Angelson & Betz 1997). These times scales have been consistent for HRP and FM1-43. However in the case of FM1-43 the rate of dye partitioning from the membrane was 2.5 sec (determined by its time constant). At the frog NMJ it was found that in the case of treatment with a Protein Kinase inhibitor there was no uptake of the dye FM1-43 but there was a release of neuro-transmitter thus confirming that there is a pathway that is faster than that required for FM1-43 to partition to the membrane. FM dyes with different time constants have been used recently and these enable an analysis of differential rates of vesicle replenishment at the synapse. For example FM2-10 is relatively hydrophilic and therefore washes off quickly as compared to FM1-43 or the red dye FM4-64. Also FM2-10 washes off from the deep narrow mouthed membrane invaginations and FM4-64 and FM1-43 does not. When a solution containing a mixture of red and green dyes was used at the synaptic terminal it was found that the FM4-64 remained in invaginations and labeled the periphery of the nerve terminal away from the active zone and the both the dyes labeled internalized regions nearer to the active zone (Richards et al. 2000). Thus the vesicles, which are in agreement with the Heuser model, were labeled red and the vesicles, which are labeled yellow, were in agreement with the Ceccarelli model of synaptic recycling. Also when a brief stimulus (2 min after loading) was applied to release the labeled vesicles it was found that 90% of the vesicles which were released were FM2-10 labeled and only 20% of the vesicles were released at that time. Recently it has been found that there might be vesicles, which do not leave the active zone and are refilled and ready for release immediately. They do not leave the membrane of the active zone and truly function to conserve the exocytic machinery and therefore were named 'kiss and stay'. This pool of vesicles, which seems to be resident at the active zone, participates in 'kiss and run' or 'kiss and stay' synaptic vesicle cycle, and constitute the readily releasable pool of vesicle (Pyle et al. 2000).

While a 'kiss and run' model has quite some interesting implications like being able to recycle fast on high stimulation rates, the very absence of mechanisms of actualization beg new thoughts to explain some of the observed results. It is equally likely that such effects assayed in mutants are induced rather than inherent. They reflect properties of vesicle membrane tending to maintain vesicles as vesicles. Specific caveats arise from use of different mutations and levels of precision in different synaptic and non-synaptic contexts. A heightened release could well leave a large fraction of vesicle membranes fused with the pre-synaptic membrane and due to the inherent property of vesicles to bud back and cause ballooning of contiguous vesicle membranes. This could show up as positive signals for endocytosis seen by FM-143 uptake but inability of downstream mechanisms to cope with replenishment of vesicular contents may result in synapse failure. On the other hand, where such structures do not go through specific Clathrin

/ Adaptin / Endophilin mechanisms that may have a role in precision but are still competent to be filled with neurotransmitter, it may result in variable quantal size and smaller quantal content in subsequent rounds of exocytosis. Some of these ideas are illustrated in figure 6.

Reserve and Readily Releasable Pools of Vesicles

The two pathways for endocytosis, that arise at different intensities of stimulus operate at different levels of intracellular calcium and seem to fill different pools of vesicles. Several studies suggest that the fast pathway fills the more active pool of vesicles, which is preferentially, released at the active zone and the slow pathway fills the reserve pool, which is more centrally located. An elegant description of the two pathways has been achieved by the recent application of a GFP maturing protein. It was shown that vesicles which are newly formed become a part of the readily releasable pool of vesicles and vesicles which are formed later become a part of the reserve pool (Duncan et al. 2003). The reserve pool function comes into play when high frequency stimulus is given. There have been studies, which label either pool preferentially and also molecular mechanisms, which mobilize the reserve pool to join the readily releasable pool. Birks & MacIntosh 1961 first suggested that releasable acetylcholine in the cat cervical ganglion is compartmentalized into two pools, a small readily releasable pool (RRP) and a larger less-releasable one. Since then, two pools of synaptic vesicles have been documented in various preparations.

Greengard's group was the first however to show that Synapsin labeled pools of vesicles differentially at the synapse with a distal pool labeled by Synapsin and a proximal pool devoid of Synapsin and present near the active zone (Pieribone et al. 1995). Pre-synaptic injection of Synapsin antibodies results in the disappearance of the distal pool leaving the proximal pool unaffected and resulting in synaptic depression when subjected to high frequency stimulus. This suggested that the presence of reserve pool was required for sustained stimulation.

The readily releasable pool has also been defined as that which is released by a hypertonic shock in hippocampal synapses. It has been found that when hypertonic shock is applied there is a release of vesicles and quantal content recrudesces to initial value with a time constant of 10 sec. The quantal size of the readily releasable pool is 10 quanta. It has been found that phosphorylation of Synapsin by Calcium and Calmodulin Dependent Kinase, MAP Kinase and Calcineurin regulates the number of vesicles at the readily releasable pool recruited from the reserve pool (Chi et al. 2003).

In Munc 13 mutants as mentioned in the above sections it was found that the docked pool of vesicles is unaltered but there is a decrease in the amplitude of hypertonic release. This means that the docked pool of vesicles undergoes a Munc13 modification to become primed to release and hence there are really three pools of vesicles at the synapse. These are: a reserve pool present away from the active zone, a docked pool of vesicles present at the active zone and a finally a pool at the active zone which has already been primed and undergoes fusion upon stimulus. We hypothesize that the readily releasable pool of vesicles corresponds to the primed vesicles. The docked pool allows for fulfilling the Katz criterion of constancy of quantal content.

Sub-cellular Localization of Endocytosis: Hot Spots

Exocytosis which results in the release of the neurotransmitter as discussed in preceding discussions occurs at the active zone. Electron microscopic studies mentioned above have been interpreted to suggest that endocytosis occurs in two ways, the fast pathway that is at or close to the active zone and a slow one effective at high intensity stimulus and relatively low calcium operating away from the active zone. It is now becoming increasingly evident that endocytic retrieval of vesicles at the synapse may also be restricted to specialized zones. "Hot Spots" for endocytosis were first suggested from studies of sub-synaptic distribution of Dynamin in relation to other markers in *Drosophila* NMJ's (Estes et al. 1996). This has been reiterated by the reticulate pattern of staining obtained with antibodies to Dynamin and glutamate receptor (Roos & Kelly 1999). Recent experiments carried out in cultured mammalian neurons elegantly confirm the functional existence of such 'Hot Spots' for endocytosis. Kymographic analysis of confocal microscopic images of GFP tagged Clathrin show occurrence of assembly and disassembly rapidly and repeatedly at 'Hot spots'

throughout dendrites and at the tips of dendritic filopodia in young neurons (Blanpied et al. 2002). These zones are typically located adjacent or lateral to the postsynaptic density (PSD), which marks zones of exocytosis. What molecules or structures may organize these hot spots is still a big question. In *shi*^{ts1} NMJ terminals depleted of synaptic vesicles, vesicle membrane proteins are relatively evenly dispersed on the pre-synaptic plasma membrane (van de Goor et al. 1995). This strongly suggests that cargo molecules do not define the endocytic zones. It appears that the components of the endocytic machinery itself could act as spatial organizers. The GTPase Dynamin, α-Adaptin, lap (AP180), DAP160 and stoned at the *Drosophila* neuromuscular junction are present at relatively sharp concentrated 'hot spots' in the plasma membrane (Roos & Kelly 1999, Zhang et al. 1998, Gonzalez & Jackle 1997, Stimson et al. 2001, Fergestad & Broadie 2001). In addition such endocytic 'hot spots' exclude active zones (Roos & Kelly 1999). Other molecules, which are likely to be involved in endocytosis, have also been detected in hot spots at the frog and fly neuromuscular synapses. Molecules such as Endophilin which have been known to be recruited to these zones in the presence of calcium have been shown to be localized to such hot spots (Rikhy et al. 2002, Guichet et al. 2002). These observations suggest that vesicle internalization at least by one of the pathways occurs at sites where the endocytic machinery is constantly available.

It has been found that after dense core granule cell exocytosis, fast internalization is Clathrin independent and Calmodulin dependent while slow endocytosis in contrast is Clathrin dependent and Calmodulin independent (Artalego et al. 1996, Peters & Mayer 1998). This has led to a suggestion that these two pathways may be mechanistically distinct but identification of molecular compo-nents, which serve only one pathway, has been difficult. Interestingly however, it is known that in *Drosophila shi*^{ts} mutants, at least in the nerve terminals, all endocytosis is blocked at restrictive temperatures suggesting that both the pathways require Dynamin. The requirement of Clathrin however has not been demonstrated in both the pathways.

Other molecules which can potentially control the spatial organization of machinery and signaling at the synapse include molecules such as phosphatidyl inositol bisphophate (PIP2). In non-neuronal cells PIP2 has a non uniform raft like distribution (Martin 2001). The initial stages of Clathrin coat formation depend upon PIP2 mediated recruitment of core Clathrin coat and coat-associated proteins such as AP2, AP180 and Epsin (Gaidarov & Keen 2001, Ford et al. 2001, Itoh et al. 2001). Many proteins have PH domains, which bind to PIP2. The binding of PIP2 to the PH domain of Dynamin is crucial for its recruitment and further hydrolysis of PIP2 by Synaptojanin which is imperative for coat removal (Hinshaw 2000, Mcpherson et al. 1996). An injection of peptides that inhibit Synaptojanin function shows accumulation of Clathrin coated vesicles (Gad et al. 2000). Finally synaptic vesicle components to be internalized may be present in membrane 'rafts'. It has been shown in studies on PC12 cells that the formation of synaptic like micro vesicles was reduced by cholesterol depletion without affecting fluid phase uptake (Martin 2000). Thus while there are tantalizing clues and indications as to the sites of endocytosis, precise identity of components and details of organization of the 'Hot Spots' are far from clear. Refer to table 2 for a summary of proteins found to be a part of endocytic hot spots.

Calcium Sensor for Endocytosis

Calcium triggers exocytosis at the pre-synapse. This is through entry of calcium via voltage gated calcium channels at the nerve terminal. This causes the increase in calcium in a small micro-domain of 10-100 μM. Endocytosis or vesicle recycling is closely coupled to exocytosis and consequently a modulation of vesicle recycling by calcium levels is an expected possibility. Alteration of the levels of calcium at the nerve terminal changes the mode of synaptic vesicle recycling as mentioned previously. The first direct demonstration of the requirement of calcium in endocytosis was at the frog synaptic terminal where when calcium independent exocytosis was stimulated by black widow spider venom endocytic recycling did not proceed (Clark et al. 1972). In chromaffin cells and retinular bipolar neurons 'kiss and run' mechanism operates preferentially at high calcium concentrations (Ales et al. 1999, Neves et al. 2001). In the lamprey giant synapse the retrieval of synaptic vesicle membrane is blocked in the absence of calcium and restored by adding low levels of calcium (Gad et al. 1998). This

is also seen at the *Drosophila* neuromuscular junction where recycling events require calcium. However recovery from the *shibire* block is calcium independent since the calcium requirement precedes the *shibire* block (Ramaswami et al. 1994). Thus calcium is needed at a distinct and early step in endocytic retrieval of synaptic vesicle recycling. At the synapse Calcineurin is hypothesized as a sensor for endocytosis. Internalization of calcium causes the activation of Calcineurin, which in turn de-phosphorylates various proteins such as Dynamin, Synaptojanin and Amphiphysin. The phosphorylation cycle is essential for endocytosis. Protein kinase C is known to phosphorylate Dynamin. Phosphorylated Dynamin and Adaptin are localized to the cytosol and removal of the phosphate is in concert with assembly of coat proteins and the endocytic machinery, which catalyze the endocytic reaction (McPherson et al. 1996,Liu et al. 1994a,b, Bauerfeind et al. 1996, Wilde & Brodsky 1996). Another kinase AAK1 has been shown to bind to α adaptin and phosphorylate μ2 adaptin. This phosphorylation is inhibitory to endocytosis in neuronal and non-neuronal cells. It is quite conceivable that calcium activated dephosphorylation could relieve this inhibition. However, whether phophorylated μ2 is a substrate for a calcium dependent phosphatase has not yet been established (Conner & Schmid 2002). Calmodulin which binds calcium has been shown to be selectively important for the fast endocytic pathway (Artalego et al. 1996).

Molecular Mechanisms of Membrane Invagination or Budding

Clathrin mediated endocytosis is at least one method of recycling at the synapse and is thought to function in the slow pathway of endocytosis which occurs away from the active zone. It remains to be seen whether the alternate fast pathways have exclusive machinery or not. Identi-fication of such machinery may be an important argument against reservations expressed earlier on these being inherent mechanisms. Endocytosis of receptors at the plasma membrane and vesicles at the synaptic terminal occurs by the progressive sequential assembly of Clathrin that serves to concentrate the cargo and deform the membrane to form a bud. The bud matures and gives rise to a Clathrin-coated vesicle, which is uncoated, and fuses with endosomes or is targeted for imme-diate refilling with the neurotransmitter.

Clathrin the major component of the coat was first purified from bovine brain with its accessory proteins (Pearse & Crowther 1987, Maycox et al. 1992). Clathrin molecules are organized into a trimeric complex called the triskelion with three heavy chains and three light chains. Clathrin however does not bind to membrane on its own and needs adaptor complexes to do the same. There are different complexes which function in sub-cellular domains of the cell and it is well established that at the plasma membrane the tetrameric complex AP2 (adaptor protein complex 2) and AP180 are functionally relevant. It consists of four non-covalently bound subunits two large subunits (β2 and α Adaptin), one medium subunit (μ2 Adaptin) and one small subunit (δ2 Adaptin). During receptor mediated endocytosis the adaptor proteins bind to the tails of cargo proteins and sequester them into the endocytic vesicle. The α subunit is essential for specifying the site of Clathrin assembly by targeting AP2 complexes onto the membrane. The cytoplasmic tail of cargo proteins binds to the μ subunit or the β subunit of the AP2 complex (reviewed in Conner & Schmid 2003). This is seen at the synapse also where the C2B domain or the extreme carboxy terminal domain of Synaptotagmin has been shown to bind to the μ subunit of the AP2 complex and is also found in zones where endocytosis takes place (Chapman et al. 1998, Zhang et al. 1994). This μ2 subunit binding is however not through the tyrosine-based signal like in non-neuronal cells (Haucke & Camilli 1999, Jarousse & Kelly 2001, Littleton et al. 2001). *In vitro* binding experiments and studies in non-neuronal cells show that Synaptotagmin plays a critical role in nucleating a coated pit–containing cargo, AP2,and Clathrin (von Poser et al. 2000). The β2 subunit binds to Clathrin and is alone capable of triggering Clathrin assembly. AP180 is absolutely essential for Clathrin cage assembly on the membrane. Several proteins are known to bind the α Adaptin ear domain: Epsin, Auxilin, Eps15, Aak1, AP180, GAK, Amphiphysin and Synaptojanin. AP180, Eps15, AP180, Epsin and Clathrin are known to bind to the β2 ear domain. The additional proteins linked to Clathrin help in events of Clathrin coat formation, membrane fission, lipid metabolism and also remodeling of the

cytoskeleton (reviewed in Conner & Schmid 2003, Higgins & Mcmahon 2002). Figure 4 illustrates the salient features of Clathrin mediated endocytosis, a consensus that seems to be emerging. Table 2 is provided as an easy reference for several molecules mentioned in this section.

Clear synaptic vesicles show a uniform size at the synapse. *Drosophila lap* and C *elegans Unc11* mutants (defective in AP180) and *Drosophila stoned* mutants show an increase in vesicle size (Zhang et al. 1998, Stimson et al. 2001) thus indicating a role in fine-tuning the size of synaptic vesicles. Lap is essential for interacting with Clathrin and it has been found that Clathrin mislocalizes at the synaptic terminals of *lap* mutants. AP180 has also been shown to restrict the size of the Clathrin cages in an *in vitro* cell free system. There is an increase in quantal size indicating that the quantal amount of neurotransmitter is proportional to the size of the vesicles. In addition there is a decrease in the FM1-43 dye uptake establishing a role for AP180 in vesicle recycling. In the *C elegans unc11* mutants, Synaptobrevin is mislocalised to and enriched in axonal tracts. This might imply an indirect effect on recycling of different cargo components, as there is no evidence of direct binding between AP180 and Synaptobrevin (Nonet et al. 1999).

Stoned on the other hand is a *Drosophila* mutant, isolated in a classical screen for temperature sensitive paralytic mutants and is known to interact with *shibire*. *Stoned* mutants enhance the *shibire* phenotype of synaptic fatigue (Stimson et al. 2001, Fergestad et al. 1999). *Stoned* encodes two polypeptides Stoned A and Stoned B in a dicistronic message (Stimson et al. 1998). Stoned B has a domain which shows homology with adaptor proteins and *stoned* also genetically and biochemically interacts with *synaptotagmin* thus providing an in *vivo* evidence for Synaptotagmin role in synaptic vesicle recycling (Phillips et al. 2000). The human Stonin 2, which has homology with *Drosophila* Stoned B, also binds the C2B domain of Synaptotagmin. In addition it shows binding properties to the AP2 complex directly as well as Eps15 and Intersectin1 (Martina et al. 2001, Walther et al. 2001).

Eps 15 is an accessory protein, which binds to AP2 constitutively. Mutants of Eps 15 which have been isolated in *C. elegans* do not have a direct defect in endocytosis. However they interact with Dynamin mutants indicating a role in endocytosis (Salcini et al. 2001). Our own unpublished observations suggest that Eps15 have a defect in the size of vesicles thereby regu-lating the fidelity of synaptic vesicle recycling (Majumdar & Krishnan unpublished observa-tions). Further studies with model lipids suggest that Epsin which is the binding partner of Eps15 has a role in vesicle formation by inserting into the bi-layer and generating membrane curvature (Ford et al. 2002).

Intersectin binds components of the endocytic machinery (Clathrin, AP2, Eps15, Epsin, Dynamin and Synaptojanin), components of the Actin cytoskeleton (N-WASP and Cdc42) components of the MAP Kinase signaling pathway and components of the exocytic machinery as well (SNAP25). Thus true to its name it intersects various steps of the synaptic vesicle cycle (Zamanian & Kelly 2003, Conner & Schmid 2003). What bearing these interactions have in regulating endocytosis will be a major focus of future studies. Genetic studies in *Drosophila* will be a significant input in delineating *in vivo* correlates of these interactions. Table 2 provides a summary for proteins implicated in endocytosis with their putative roles in invagination and budding.

Molecular Mechanisms of Membrane Fission

When the assembly of Clathrin with the adaptors has sequestered a membrane compartment to be recycled it is essential to recruit machinery which will enforce fission. A critical role for the GTPase Dynamin has been outlined beyond doubt by *Drosophila shibire* mutants, which paralyze at restrictive temperatures (Grigliatti et al. 1973). In these mutants there is no defect in vesicle exocytosis but the synapse finally depletes when there are no vesicles to refill due to a lack of recycling thus resulting in a lack of synaptic transmission at the nerve terminal. Kosaka and Ikeda found that recycling intermediates accumulate at the synapses of *shibire* and this led to the initial suggestion of its role in endocytosis (Kosaka & Ikeda 1983). Molecular cloning of this gene led to the suggestion that the GTPase Dynamin is involved in endocytosis (van der Bleik & Meyerowitz 1991). Since then there have been streams of papers, which indicate a role for Dynamin in endocytosis in different organisms and cell lines (reviewed in Sever et al. 2000). Dynamin is

a multi-domain protein which contains an N terminal GTPase domain, a middle domain, a GTPase effector domain (GED) which serves as an internal GTPase activating factor, a PH domain which enables binding to lipids through phosphatidylethanolamine bisphosphate and a proline rich domain which binds and probably recruits a whole host of proteins at the site of endocytosis. Dynamin forms oligomers in different salt concentrations and GED and GTPase domain are important for this oligomerisation. The GTP hydrolysis of Dynamin has been found to change significantly upon oligomerisation, binding to microtubules, phospholipids, phosphoinositol and SH3 domain containing proteins (Hinshaw Schmid 1995). Dynamin unlike other classical small GTPases has a high rate of GTP hydrolysis and a low affinity for binding GTP (Hinshaw 2000). Our studies in *Drosophila* identified Nucleoside Diphosphate Kinase as an exchange factor, which converts inactive GDP-Dynamin to active GTP-Dynamin (Krishnan et al. 2001, Palacios et al. 2002, Baillat et al. 2002). Calcineurin dependent dephosphory-lation and PKC dependent phosphorylation is an important regulating mechanism for loading Dynamin onto the membrane (reviewed in Cousin & Robinson 2001, Liu et al. 1994a, Lai et al. 1999). The 'Dynamin cycle' shown in figure 5 summarizes the variety of modifications and structural changes the protein Dynamin undergoes during endocytic retrieval of synaptic vesicles.

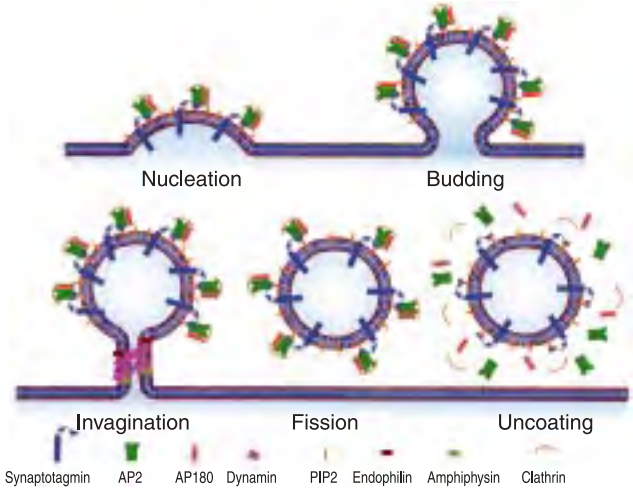

Figure 4. Mechanism of Clathrin mediated Membrane Fission. It is likely that any regular arrangement of proteins on the surface, including synaptic vesicles is targeted by the Clathrin machinery for endocytosis. This process is separated, based on available literature into nucleation, budding, invagination, fission and uncoating. Nucleation of the Clathrin coated machinery at the endocytic 'hot spots' is a consequence of calcium mediated dephosphorylation and attachment to PIP2 sites on the plasma membrane. AP2 binds to Synaptotagmin which can serve either as the cargo or as a signal for nucleation. AP180 and AP2 recruit Clathrin to form the Clathrin coat which subsequently results in budding of the plasma membrane. Proteins like Dynamin and Endophilin facilitate the actual fission event. It is likely that the integral proteins of the vesicles that were formerly part of the 'fusion pore complex' in a reversal of roles turn in to 'fission scars' which will later disperse by diffusion. These fusion pore proteins may in fact also be the elements which recognize Endocytic 'hot spots' that are rich in Adaptin and Dynamin. These 'hot spots' may serve as a general scaffold, where along with Clathrin assembly they nucleate activities like that of Endophilin at the periphery of invaginating vesicles. The Endocytic machinery then is only involved in regulating curvature in a restricted region marked exclusively by vesicle components. Uncoating of Clathrin from the vesicle is accomplished by Synaptojanin, Auxilin and Hsc70.

Dynamin coats synaptosomes, liposomes and lipid nanotubes via its PH domain. GTP hydrolysis has been found essential for fission of the vesicle (Takei et al. 1995, Takei et al. 1999, Stowell et al. 1999, Sever et al. 1999). Collared pits accumulate in *shibire* mutants at restrictive temperatures suggesting a ring like arrangement for Dynamin oligomers at the vesicle neck. There are several hypotheses of the role of GTP hydrolysis by Dynamin in mediating actual fission. Dynamin was found to coat synaptosomes and GTP hydrolysis was found to be congruent with vesiculation. Further Dynamin was found to tubulate liposomes and this tubulation is enhanced in the presence of Amphiphysin (Takei et al. 1999). When GTP was provided these vesiculate. It has also been demonstrated that Dynamin can oligomerise into long rings around lipid nanotubes and the conformation adopted by GTP bound Dynamin versus GDP bound Dynamin was different leading to a model where GTP hydrolysis and subsequent change of confirmation would lead to a mechanochemical force generation which would pinch or pop off the vesicle (Stowell et al. 1999, Sever et al. 2000).

Dynamin binds to several SH3 containing domains via its PR domain and these regulate endocytosis. Among these are Amphiphysin, Endophilin, Intersectin, Grb2, Src and Syndapin (Simpson et al. 1999). Table 2 provides a list of some of the proteins which promote fission in concert with Dynamin. Loading of Dynamin onto the vesicle neck has to be by a protein that interacts with the adaptor protein complex as well as Dynamin.

Amphiphysin has been one such candidate molecule. Two Amphiphysin genes have been identified and Amphiphysin1 is neuronally expressed. Different splice variants exist in different tissue types indicating a broader role in endocytosis. It contains three domains: N terminal coil coiled domain, a middle PR domain and a C terminal SH3 domain. These domains enable an interaction at the N terminus with Clathrin and Adaptin and the C terminus with Dynamin, Synaptojanin and Endophilin. It may also interact with AP180. In the lamprey giant synapse injection of dominant-negative peptides against the SH3 domain binding region which interacts with Dynamin results in a use dependent accumulation of budding vesicles at the synaptic terminal. Amphiphysins also run through a phosphorylation and de-phosphorylation cycle and this might be essential for nucleating endocytic molecules (Cousin & Robinson 2001). Phosphorylation of Amphiphysin decreases its affinity for all essential for endocytosis. *Drosophila* Amphiphysin mutations surprisingly have no defect in synaptic vesicle recycling and it is in fact localized to the post synapse. Amphiphysin mutants show a mild defect in locomotion (Razzaq et al. 2001). This makes it difficult to establish a role for Amphiphysin in endocytosis.

Endophilin has been another attractive candidate, which can facilitate fission of the membrane (Ringstad et al. 1999). It has an SH3 domain and can therefore be recruited to vesiculating membrane Dynamin. It can also bind Synaptojanin. It can directly bind and tubulate lipids and it has an appropriate lipid modifying activity that is essential for altering the curvature of the membrane. It is a Lysophosphatidic Acid Acyl Transferase that adds unsaturated long chain fatty acids to lysophosphatidic acid (Schmidt et al. 1999). This converts cone shaped lipids to inverted cone shaped lipids thus enhancing curvature of the membrane and if this takes place at the neck of the invaginated pit it is capable of producing fission. A clear role for Endophilin in vesicle recycling was shown in studies in *Drosophila* mutant animals that had lowered levels of Endophilin (Verstreken et al. 2002, Rikhy et al. 2002, Guichet et al. 2002).

Thus while several suggestions have been made for a pure mechano-chemical role for Dynamin either pinching or pushing with sheer physical force (reviewed in Sever et al. 2001) we suggest a broader scheme in which Dynamin provides a scaffold for organizing constituents of the fission machinery which may be simply a getting together of several integral membrane proteins and concerted sorting of the vesicular versus surface proteins and at the same time providing sites for molecules like Endophilin that have the right biochemical activities to effect curvature changes needed for precise and fast fission processes. By the very nature of membrane constituents it cannot be denied that many such activities could be dispensed with in much slower, non regulated endocytic events and such may happen at faster speeds in unusual circumstances as we discussed earlier arising from high frequency stimulation or recovery from paralysis in *shibirets* flies.

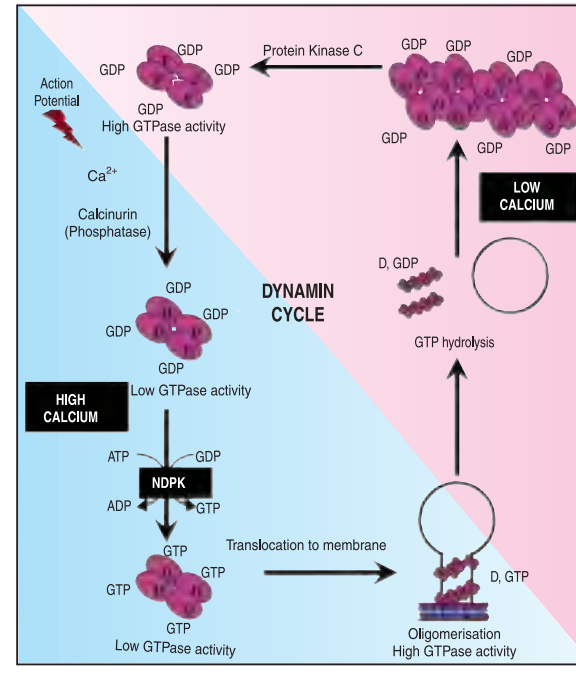

Figure 5. The Dynamin cycle. Dynamin cycles between membrane bound and cytosolic, phosphorylated and dephosphorylated and GDP bound and GTP bound forms. The figure portrays the diverse forms and fits them in to the Endocytic process taking in to account the range of biochemical results that are referred to in text. Dynamin is proposed to exist predominantly in phosphorylated GDP bound tetrameric forms; there is no loss of generality if they are monomeric. Dephosphorylation of GDP bound tetrameric Dynamin invests it with competence to accept NDPK and convert to GTP bound form. GTP bound tetramers translocate to endocytic 'hot spots' on the membrane and oligomerise around the neck of invaginating vesicles. GTP hydrolysis disengages GDP bound Dynamin from budded vesicles. The oligomeric GDP bound Dynamin is converted by phosphorylation to tetrameric GDP bound form. Alternatively tetrameric GDP bound Dynamin is converted to monomeric GDP bound form, thus completing the cycle.

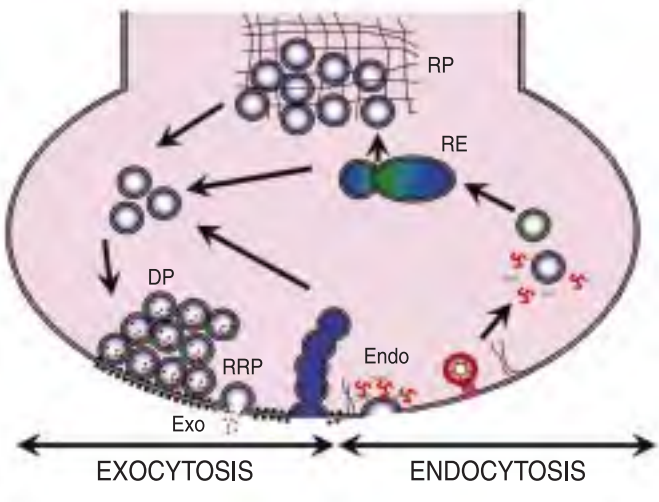

Figure 6. Pathways of Synaptic Vesicle Cycle. Vesicles are possibly forcibly retained on the target membrane and would tend to balloon inward when there is a large fraction remaining un-retrieved. The recycling machinery may not distinguish a large mass of vesicular membranes on the target membrane from the presumptive endosomes that result from fusion of budded vesicles. This may thus lead to an induced alternate pathway. It is also possible such entities are competently filled with neurotransmitter. This view is intuitive and does not demand any distinct machinery and would account for most of the results.

Clathrin Uncoating Mechanisms

The Clathrin coat has to be removed for vesicle reuse and subsequent fusion. Several proteins regulate uncoating of the vesicle. The phosphoinositide metabolism has been implicated in regulating uncoating of Clathrin monomers of the newly formed vesicle. Synaptojanin is a phosphoinositide phosphatase and can bind Amphiphysin, Endophilin, Dynamin and AP2. Synaptojanin can result in modulation of phosphoinositide 4, 5 bisphosphate which is known to preferentially bind many endocytic molecules. In mice and C *elegans* mutants for Synaptojanin an accumulation of coated pits is seen and there is a subsequent block of synaptic vesicle recycling. This suggests that Synaptojanin is specifically required for regulation of removal of coat components.

Auxilin and Hsc70 are required for actual uncoating of Clathrin coated vesicles (Ahle & Ungewickell 1990, Schlossman et al. 1984, Ungewickell et al. 1995, Pishavaee et al. 1997). Auxilin contains a Clathrin binding domain and a J domain which binds Hsc70. It also interacts with AP2. Approximately 20 years ago a protein with properties of uncoating Clathrin coated vesicles was purified in *vitro*. This was found to be a chaperone with an N terminal ATPase and identified as Hsc70. Hsc70 is capable of uncoating and this activity is greatly enhanced by J domain containing proteins such as Auxilins. Due to these activities it has been suggested that Auxilins can potentially recruit Hsc70 and this is responsible for uncoating. Inactivation of yeast and nematode Auxilin has adverse effects on Clathrin mediated endocytosis (Pishavaee et al. 2000, Greener et al. 2001).

Figure 7 summarizes the crucial molecular players in membrane trafficking mechanisms in synaptic vesicle cycle. Here again we wish to point out that changes in protein- protein interactions facilitated by two dimensional mobility on membranes may be crucial for fission as well. The molecular interaction may facilitate curvature changes and, specificity of cargo and precision of vesicular size while fission itself may involve a reversal of events relating to interaction of some integral protein components from what is indicated for exocytosis in figure 3.0.

The Role of Cytoskeleton in Endocytosis

Vesicles after undergoing fission through the membrane are encountered with a meshwork of the cytoskeleton and it is thought to be essential for directing the vesicle to appropriate positions in the cell. In yeast the Actin cytoskeleton has been found to be essential for endocytosis (Fehrenbacher et al. 2003). But in the case of mammalian cells and especially neurons application of Actin depolymerising drugs has little or no effect on any part of the vesicle coat formation or movement (Morales et al. 2000). However it is likely that Actin plays a significant role in formation of endocytic hot spots or in propelling nascent vesicles. Actin filaments are known to dissociate and reassociate during synaptic vesicle cycling (Bernstein et al. 1998). In the frog neuromuscular junction F-Actin and beta-Fodrin are concentrated in non release domains (Dunaevsky & Connor 2000). Recent EM studies in lamprey giant synapse show a stimulus dependent reordering of Actin filaments (Shupliakov et al. 2002). Immuno fluorescence studies in hippocampal synapses indicate that different pools of Actin might be present in release and recycling zones. Different proteins such as Munc13, RIM, CAST, Bassoon and Piccolo have been found to be present associated with the cytomatrix at the active zone (Shapira et al. 2003).

Recent studies using evanescent wave microscopy suggested that Actin loading is spatially and temporally coordinated with Dynamin recruitment to the necks of the coated vesicles (Merrifield et al. 2002). Dynamin has also been known to localize at Actin comet tails. In neurons it has been seen that Latrunculin A treatment during the first week of the cell culture results in the drastic changes in synaptic ultra structure and in complete loss of vesicle clusters and synaptic vesicle recycling activity. However in the case of mature neurons these vesicle clusters were largely resistant to the treatment (Zhang & Benson 2002a). This suggests that formation of synapse vesicle clusters is largely independent of Actin cytoskeleton in mature neurons.

Recycling Compartments

Do synaptic vesicles go through recycling endosomes at the synapse just like other cells during trafficking of receptors? Lysosomes have not been found in mature synapses in the central nervous system of higher vertebrates. They are only found in growing neurons and also in certain neurodegenerative conditions. Although lysosomes are generally not observed at mature synapses in the CNS, lysosomes have been observed at newly formed pre-synaptic terminals and are observed to accumulate within pre-synaptic terminals in lysosomal storage and neurodegenerative disease (Broadwell & Cataldo 1984, Parton et al. 1992, Nixon & Cataldo 1995). Furthermore, there is evidence for lysosomes within the axon that appear to concentrate to the nodes of Ranvier (Gatzinsky & Berthold 1990, Overly & Hollenbeck 1996). In other studies, a synaptic compartment considered to be pre-lysosomal has been described that may have a limited capacity for protein degradation (Nixon & Cataldo 1995). Endosome like organelles are a part of the slow recycling pathway at the synapse which occurs during high frequency stimulation. *Drosophila* mutants in Dor and Car which localize to large multivesicular late endosomes have a defect in endosomal degradation but have no conceivable defect in synaptic transmission and only seem to regulate synapse size (Sriram et al. 2003, Narayanan et al. 2000). Spinster is a *Drosophila* protein which labels late endosomal or lysosomal compartments. Spinster containing organelles have been located at the synapse in a recent report and mutants in this protein result in an increase in synaptic growth (Sweeney & Davis 2002). Behavioral experiments involving conditional knockout of endocytosis and exocytosis suggest roles for fusion events after endocytic retrieval. It is tempting to suggest that these indicate formation of sorting endosomes. It is quite conceivable that neurotransmitter packaging may occur in bulk in endosome like structures from where uniform transmitter filled vesicles are pinched off and become part of the reserve pool (figure 6). This will be particularly useful in minimizing errors of composition.

Perspectives

A broad picture of vesicle cycle that we have presented (figure 7) highlights a consensus although several features are yet to be ascertained. The model tries to accommodate views and results without details of individual molecules or their activity. The following points that relate to the questions raised earlier are worth reiterating. We do think it is possible to address these in the future.

Sites for Exocytosis and Endocytosis

Distinct sites of fusion and fission enhance the precision and speed of synaptic vesicle cycle. While the 'active zones' for exocytosis are very well characterized, the endocytic 'hot spots' have convincingly been shown only recently. Definition

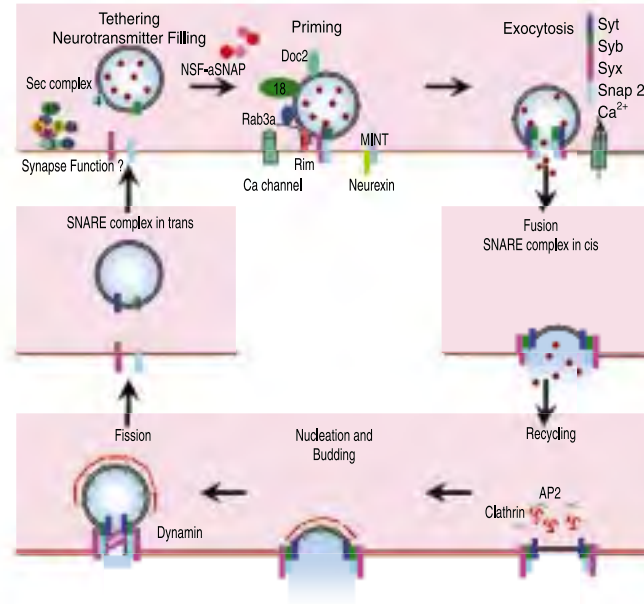

Figure 7. Molecular players of the Synaptic Vesicle Cycle. A pictorial summary of the major components of the synaptic vesicle cycle namely exocytosis and endocytosis is presented. All of the molecular players that have been confirmed to play a role in either of these processes at the synapse are indicated.

of endocytic 'hot spots' and their molecular components is likely to be an area of intense research. We suggest that some unusual pathways like 'kiss and run' may result from acute stress to the vesicle cycling machinery when having to deal with a large amount of vesicular membrane fused to the surface membrane. Identification of sites and mechanisms alone will confirm their existence as genuine alternatives to complete fusion and regulated retrieval of vesicles.

Machinery Involved in Exocytosis and Endocytosis

The machinery for fusion and fission are clearly different. The balloon analogy suggested earlier implies a reversal of roles in fusion versus fission but the molecular mechanisms are possibly not just in reverse order. Membrane components will need to be modified transiently in such a situation. In particular, curvature changes can be facilitated by lipid modification and protein-protein associations altered by reversible modifications like phosphorylation. An interesting possibility is that synaptic vesicles are retrieved by Clathrin mediated mechanisms primarily due to their ordered structure much like cross-linked receptors. Endocytic mechanism then could be broadly similar with distinctions arising because of time scales and order and composition of the endocytosed components. Both fusion and fission would involve clustering and resolving specific integral membrane proteins and restructuring, either by biochemical modifications or lateral diffusion of lipid components, to sites of fusion and right curvatures.

Cyto-skeletal rearrangements will be needed for regulating both fusion and fission. Integral membrane proteins that could diffuse and re-organize to form fusion pores and fission centers need to be identified. These may be part of both vesicles and pre-synaptic membranes and are likely to be discovered as molecules that are components of or interact with elements of endocytic 'hot spots' as well as active zones.

Triggers for Synaptic Exocytosis and Endocytosis

Primarily docking and priming organize vesicles in to a state ready to fuse at an appropriate signal, in this case a surge of calcium. Vesicles once fused will be held back from being internalized by clamp mechanisms in order that they are completely and accurately retrieved. What mechanisms assure the fidelity of retrieval of the vesicle in its entirety is not known. Calcium increase is a signal for both fusion and fission although calcium levels change only prior to release. Whether differing levels of calcium are perceived as signals for fusion and fission or the kinetics of fusion and fission processes dictate their specific order is as yet unclear. Fusion results in addition of vesicle components to the membrane. These molecules can themselves serve as triggers for invagination. It is conceivable that many molecules serve this purpose but these remain to be identified.

Mechanism and Order of Action of Molecules

Several molecules may function in concert to induce structural changes needed for fusion and fission. Many other molecules along with motor systems may be needed to modify cytoskeletal elements to clear sites and move vesicles to the membrane on the membrane and out of it after fission. The dynamics of association of these molecules will need to be ascertained in *vivo*. These events while sharing some common features may be distinctly different in different organelles. In some cases of internal trafficking, clearing cytoskeletal elements may not be a necessity. In yet other instances like in the case of synaptic vesicles the specificity may be for entire macromolecular assemblies being taken in together. SNAREs may be distinct in different contexts and it is unlikely components like CSP have any role in processes other than synaptic vesicle endocytosis. Rabs may play key roles in marking vesicles destined for different fates. While many insights have come from biochemical and *in vitro* studies, a clearer understanding of the mechanisms will be aided by physiological and cellular studies.

Major progress in genetic studies of defined systems and use of microscopic techniques like evanescent wave to study synaptic events in animals expressing specific GFP proteins in mutant and normal backgrounds can be expected in the future. Acute conditionality, like temperature sensitive loss of function in mutants, will be invaluable to assess both physiological and cellular roles of macromolecules. Some will be identified by mutations that cause synaptic failure while others

may be assessed by cellular and physiological behavior of mutations induced in homologues of molecules identified in yeast secretory and vacuolar protein sorting pathways. Fluorescence microscopy is likely to continue as the mainstay of high resolution analysis of endocytic and exocytic events and delineating the transient complexes of macromolecules that execute the fusion and fission processes. Biochemical and structural analysis of several protein molecules identified to play roles in endocytosis will be crucial to our understanding these processes in great detail. This will also allow *in vitro* analysis of many of these functions. Development of *in vitro* preparations and methods to study release and recycling with such preparations will be invaluable. To provide mechanistic details one will need to work out energetics of these processes and this will be aided by single molecule studies and studies on model membranes.

We appreciate the fact that what we have compiled so far is far from a complete description of the processes and has been somewhat biased towards genetic studies of synaptic vesicle recycling. We have been unable to do justice to a vast amount of work in other systems and methods. Many of the components of the pathway may be peculiar to the synapse and we could be missing much in taking the detour. We however hope that this review conveys the idea that the synaptic vesicle cycle is one area of cellular function that will be exciting for a long time with the promise of being completely understood in structural and functional detail.

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