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MEDICAL REVIEW

Neurotransmission and the Synaptic Vesicle Cycle

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THE NEURON DOCTRINE

The vertebrate central nervous system has evolved into a specialized organ responsible for higher cognitive and functional behaviors. Fast, reliable, stereotyped relays of messages from one neuron to the next is responsible for both intrinsic homeostatic functions such as breathing, to much of the responses necessary for adaptation and higher neuronal functioning. The concept of neurotransmission was revolutionized in the second half of the nineteenth century by the seminal work of the neuroanatomist, Santiago Ramon Y Cajal. Using new stains for microscopy and impeccable observational skills, Cajal was the first neurobiologist expanding the cell theory, introduced by Schleiden and Schwann in the 1830s, to suggest that the nervous system was a collection of diverse cellular entities that communicated with each other through non-continuous connections. These connections were termed synapses by Charles Sherrington in 1897, after the Greek word for "clasp" [1]. Thus the neuron doctrine, which holds that the nervous system is comprised of individual cells, termed neurons, gained fervor in the scientific community. Cajal's belief was in contrast to the one popularized by his famous contemporary, Camillo Golgi, whose reticularist model contended that the nervous system was one big syncycium of interconnected parts with a directly communicating protoplasm [1].

In fact, the neuron doctrine has been unequivocally supported by rigorous experimentation, with the ultimate proof provided by electron microscopic studies of the synapse by George Palade and others in the mid-1950s [1-3]. Interestingly, it has been determined that certain few neurons directly communicate through specialized intercytoplasmic junctions, termed gap junctions, which allow for bi-directional flow of ions and small signaling molecules [1, 4-6]. The function of these electrical synapses, however, is mainly thought to be the synchronization of activity within certain groups of neurons [1, 7, 8].

Since the establishment of the neuron doctrine, it has been determined that the central nervous system is comprised of roughly

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[b]*Abbreviations:* GED, GTPase, effector-domain; LPA, lysophospatidic acid; LPAAT, lysophospatidic acidacyl transferase; PIP_2, $PT_{4,5}P$; PIP_3, $PI_{3,4,5}P$; PH, Pleckstrin-homology; MEPPs, mini-end plate potentials; PA, phosphatidic; SNARE, soluble N-ethylmaleimide protein receptor; tSNARE, target SNARE; vSNARE, vesicle SNARE.

10^{11} cells of more various types than any other organ known [9]. These cells participate in up to 10^{15} synaptic connections. Furthermore, more of the primate genome is believed to be active in the central nervous system compared with any other organ [10]. This, in part, reflects the complex underpinnings of neuronal function.

THE SYNAPSE

The synapse is the principal computational unit of the nervous system. It has long been known that neurotransmission relies on the propagation of electrical impulses. In fact, there are four principal ways that neurons may communicate: a chemical interaction mediated by small neurotransmitter molecules secreted into the synaptic cleft between two neurons; an electrical interaction mediated by diffusion of an electrical impulse between two neurons; ephaptic interactions mediated by electrical field effects of closely apposed neurons; and regional interactions mediated by release of chemical and gaseous signaling molecules. This discussion will primarily deal with the chemical synaptic interactions, which are by far the most dominant in the mammalian nervous system.

The chemical synapse is comprised of a presynaptic nerve terminal, a 20 to 40 nm synaptic cleft maintained by intercellular adhesive contacts, and a postsynaptic nerve terminal [11, 12]. Synapses are most often asymmetric intercellular junctions between two communicating excitable cells, typically neurons, or neurons and muscle cells [12]. Synaptic connections arise during development through a complex and poorly understood mechanism of axonal pathfinding and target recognition underlying the intricate circuitry of the nervous system [13]. The synapse is so structurally robust, that "synaptosomes" containing only the presynaptic and postsynaptic terminals connected via the synaptic cleft can be biochemically purified [14-17].

The morphological hallmark of a presynaptic terminal, or bouton, is the presence of a dense sub-plasmalemmal cytomatrix around which exist tens to hundreds and, in some instances, up to thousands of small 35 to 50 nm membrane-bound vesicles loaded with neurotransmitter molecules (Figure 1) [12, 18]. The active zone, a term coined by Couteaux and Pecot-Dechavassine, defines the release site of the neurotransmitter-containing vesicles [19]. Occasionally, a nerve terminal may contain more than one active zone with distinct postsynaptic contacts [12]. The terminal bouton also contains cytoskeletal elements, which are believed to be involved in the generation and organization of this specialized compartment. The postsynaptic terminal also contains a dense sub-plasmalemmal cytomatrix, termed the postsynaptic density, in which the cognate receptors of the neurotransmitter molecules are clustered [12].

Chemical synaptic transmission begins with the propagation of an electrical depolarizing impulse, termed the action potential, down the axon to the nerve terminal. At the nerve terminal, the depolarizing impulse opens voltage gated calcium channels localized at or near the active zone, which then allow an increase in the local calcium concentration by up to four orders of magnitude. The increase in cytosolic calcium triggers fusion of the synaptic vesicle with the synaptic plasma membrane surrounding the active zone in a highly coupled process linking excitation with neurotransmitter release.

Subsequent to the release of neurotransmitter molecules into the synaptic cleft, neurotransmitters bind to their cognate receptors, which either directly or indirectly gate ion channels. The subsequent influx of ions through the postsynaptic ion channels generates a postsynaptic potential that then may or may not generate another action potential to continue the propagation of the signal. A given neuron may integrate the potentials generated by several presynaptic contacts enabling complex compu-

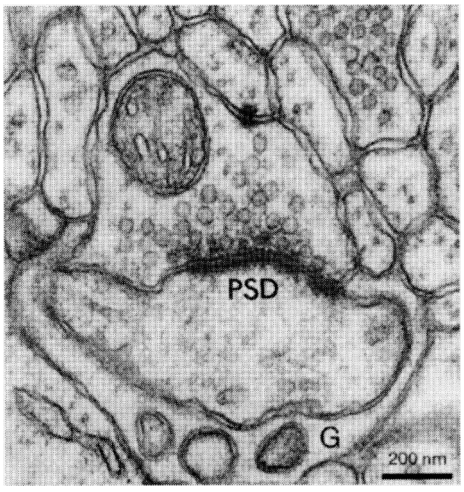

Figure 1. Electron micrograph of a mammalian central synapse. Ultrastructure of the synapse between a parallel fiber axon terminal and the dendritic spine of a Purkinje cell in the cerebellar cortex of a rat. Note the numerous synaptic vesicles adjacent to the cell membrane. PSD = post-synaptic density, G = glial cell. Originally published in [12] and reproduced with permission from Johns Hopkins University Press.

tational processing. Thus, an electrical impulse is converted into a chemical signal, which is then reconverted into an electrical impulse. By virtue of its many different regulated steps, the synapse is also the principle effector of plasticity within the nervous system, which underscores the need to functionally understand this important physiologic structure.

THE QUANTAL NATURE OF NEUROTRANSMITTER RELEASE: SYNAPTIC VESICLES

The concept that neurotransmitters were released in discrete packets, or quanta, was postulated before physical evidence of synaptic vesicles was known. Based on physiological recordings of the frog neuromuscular junction in the early 1950s, Bernard Katz and colleagues determined that there existed spontaneous stimulation independent potential changes of uniform 0.5 millivolt amplitudes [20, 21]. They postulated that these mini-end plate potentials (MEPPs)[b], as they were called, must define the minimal unit, or quantum, of neurotransmitter release to generate such a change in potential [21, 22].

In 1955, electron microscopic studies by Palay and Palade, and de Robertis and Bennett gave quantal theory a morphological correlate [2, 3]. The presence of numerous small membrane-bound vesicles present at the nerve terminal led to the suggestion that these organelles must be the determinants of quantal neurotransmitter release by fusing with the plasma membrane and releasing their stored quantity of neurotransmitter (Figure 1) [23]. It has since been found that the quantum of neurotransmitter that generates the MEPP corresponds to the amount which is loaded in synaptic vesicles of the frog neuromuscular junction, roughly five to ten thousand molecules of acetylcholine [24]. The most compelling evidence that quantal release translates to synaptic vesicle exocytosis is a study correlating amounts of released quanta measured electrophysiologically in the frog neuromuscular junction with electron micrographic images of synaptic vesicles appearing to be in intermediate stages of fusion with the plasma membrane [25-28]. Although there is little doubt, it should be mentioned, for the sake of completeness, that since no absolute physical proof for the causal relationship between vesicle exocytosis and synaptic transmission has yet been found, the vesicle theory of quantal release remains a hypothesis.

Neurotransmitter molecules released from synaptic vesicle exocytosis cross the

synaptic cleft within two microseconds, and expose the postsynaptic receptors to a concentration of 1 mM neurotransmitter, sufficient to open up to two thousand postsynaptic ion channels. This would theoretically generate a postsynaptic potential on the order of that seen with the MEPP. It has been estimated that at each release site, or active zone, an action potential has a probability of 0.3 for causing release of a synaptic vesicle (although this is likely variable and regulated at different synapses). It follows, then, that in a large neuromuscular junction containing about one thousand active zones, an action potential results in the release of roughly three hundred vesicles within 1.5 milliseconds, generating the large postsynaptic end-plate potential capable of stimulating muscle fiber excitation.

BIOGENESIS OF SYNAPTIC VESICLES

The identification of synaptic vesicles begged the next question of how and when these organelles are created. The generation of mature synaptic vesicles is thought to occur within the nerve terminal itself, compared with the direct generation from the Golgi complex typical for vesicles of regulated exocytosis. Precursor proteins and membranes, however, are formed in the endoplasmic reticulum and Golgi complex. These precursors are transported down the axon via membrane-bound tubulo-vesicular organelles through fast axonal transport. These macromolecules include the integral membrane synaptic vesicle proteins, such as synaptophysin and SV2, as well as lipid molecules [17].

A fair amount of molecular sorting occurs to generate a mature synaptic vesicle, given its compositional difference with the plasma membrane. Most evident is the virtual lack of gangliosides and a relatively large enrichment of cholesterol in synaptic vesicles when compared with the plasma membrane [17]. Cholesterol is known to be important for the structure of high curvature membranes, and depletion of cholesterol from the plasma membrane prevents efficient formation of vesicles, resulting in shallow buds that appear stunted in their ability to form high curvature [29, 30]. In addition, synaptophysin has been shown to directly bind cholesterol, potentially serving as its biochemical sorter in synaptic vesicles [31, 32].

Different lines of evidence point to the generation of a synaptic vesicle occurring at the nerve terminal [17]. First, the pleiomorphic, membranous organelles that contain the synaptic vesicle precursor proteins, do not share the same size or morphology with synaptic vesicles [33]. Second, it is known that different synaptic vesicle proteins are transported on different vesicular carriers [34], with the full complement of synaptic vesicle proteins co-existing on the same organelle only in the mature synaptic vesicle at the nerve terminal. These axonal carriers also contain other non-synaptic vesicle cargo destined for traffic to the distal axon/nerve terminal [34]. Third, neurotransmitter molecules are loaded into vesicles at the nerve terminal. Finally, it is believed that nascent transport vesicles carrying the synaptic vesicle precursors need to undergo some exo-endocytic recycling before they can load neurotransmitter molecules, implying a degree of sorting at this stage requisite for the generation of a mature synaptic vesicle. It is unclear whether some axonal endosomal sorting compartment may also be important in this process [35]. Thus, it can be thought that a synaptic vesicle is ultimately formed upon endocytosis from the plasma membrane, a fact which has important ramifications for the recycling nature of synaptic vesicles.

SYNAPTIC VESICLES EXIST IN DISTINCT POOLS

Two pools of synaptic vesicles exist at the nerve terminal: the readily releasable pool, and the reserve pool [17, 36]. Electrophysiological evidence exists for two

pools of synaptic vesicles. Upon a high-frequency train of action potentials, there ensues a burst of fast synaptic vesicle exocytosis, followed by a lower, steady-state level of release [36-39]. The burst defines the readily releasable pool of synaptic vesicles, while the steady-state is thought to correspond to a reserve pool of synaptic vesicles. This reserve pool is not immediately available for release, but is able to generate continued neurotransmission at a lower rate upon continuous stimulation, therefore highlighting the importance of this pool of vesicles during high-frequency synaptic firing. Once depleted, the readily releasable pool of vesicles is regenerated only upon a discontinuation of the excitatory train [40]. Given continuous high frequency stimulation, neurotransmission will grind to a halt as both pools of synaptic vesicles are exhausted.

Morphologically, the readily releasable pool of synaptic vesicles is thought to be those vesicles docked with the active zone in close proximity to the presynaptic plasma membrane [39, 41]. These vesicles are the ones postulated to be immediately available to fuse with the plasma membrane and release their neurotransmitter into the synaptic cleft upon stimulation [40, 42]. Alteration of the functional size of the readily releasable pool can be an important modulator of synaptic transmission and plasticity [39]. Around these docked vesicles exist many more synaptic vesicles in a cluster thought to comprise the reserve pool of vesicles [42]. While not immediately available to fuse with the plasma membrane and release their stored neurotransmitters, these vesicles are believed to be gradually recruited to the release sites at the plasma membrane where they may then dock and fuse. It is believed that the inherent delay in this recruitment process is what differentiates the readily releasable pool from the reserve pool, electrophysiologically.

There are several proteins thought to be important in the dynamics of these two pools of synaptic vesicles. The synapsins are a highly conserved protein family having activity-dependent association with synaptic vesicles [43-47]. At rest, the synapsins are clustered around the synaptic vesicles. Upon stimulation, the synapsins become phosphorylated and no longer bind to the synaptic vesicles, diffusing along the axon [48]. It is thought that this release from synapsin binding is what frees the vesicle in the reserve pool to progress to docking at the presynaptic plasma membrane [48]. Binding may then proceed to large multi-domain proteins, such as RIM, piccolo, and bassoon, which are closely associated with the active zone cytomatrix, through other synaptic vesicle proteins such as rab 3A [49]. From this "docked" site, vesicles are ready to fuse with the plasma membrane in a calcium-dependent manner to release neurotransmitter molecules into the synaptic cleft.

SYNAPTIC VESICLE EXOCYTOSIS

Due to the ability to biochemically purify a homogeneous population of synaptic vesicles, these organelles have been catalysts for the study of regulated exocytosis [45, 49]. One postulated mechanism for fusion of the synaptic vesicle membrane with the presynaptic plasma membrane occurs via the SNARE proteins [49-51]. The SNAREs comprise a diverse family of membrane-associated molecules which have the ability to bind to one another to form a robust complex having high melting temperatures and resistance to SDS denaturation [49, 52]. SNAREs have been classified into those associated with the vesicle (vSNAREs), and those associated with the target membrane (tSNAREs) [53]. In general, a SNARE complex consists of one vSNARE and two different tSNAREs [52], with the specificity of the vesicle-target membrane interaction thought to be inherent in the specific interactions of the various SNARE proteins [54]. The prototypical vSNARE is the synaptic vesicle integral membrane protein, synaptobrevin. The prototypical tSNAREs are the integral

membrane protein, syntaxin, and the lipid modified protein SNAP-25. It is generally believed that complex formation between the respective SNARE proteins of the vesicle and target membrane contains enough energy to begin a process that pulls the apposing membranes together. The energy contained within the SNARE complex is thought to thermodynamically drive the approximation of the two adjacent membranes until they mix, allowing for fusion. SNARE-mediated membrane fusion has been implicated in many forms of membrane trafficking events [54].

Two major lines of evidence support the SNARE hypothesis of membrane fusion. The first involves use of bacterial toxins from Clostridia, which selectively target various sites in the different neuronal SNARE proteins for proteolytic cleavage. These toxins potently block neurotransmission and are fatal if left untreated [55]. The block in neurotransmission lies at the level of synaptic vesicle exocytosis, with no effect on the total number of synaptic vesicles and an increase in the numbers of vesicles docked at the active zone [56]. This implicates the SNAREs to be necessary in a late stage of fusion with the plasma membrane, after docking of the vesicle to the active zone. The other line of evidence directly tests the ability of the SNARE proteins to mediate lipid bilayer fusion. Using artificial proteoliposomes containing various populations of vSNAREs and tSNAREs, Rothman and colleagues showed that the SNARE proteins are the minimal machinery sufficient to produce coordinated membrane fusion between proteoliposomes containing complementary SNARE proteins [57].

The probability of synaptic vesicle exocytosis increases five to six orders of magnitude with the influx of calcium at the nerve terminal; however, the nature of the calcium sensor in this process has remained elusive [49]. The calcium channels at the nerve terminal exist at the active zone, and generate a stimulation-dependent increase in local calcium concentration from roughly 100 nM to 100 μM . The time from influx of calcium to vesicle release can occur within 100 to 200 microseconds, indicating that the calcium sensor must reside in an area at least within 100 nm from the calcium channels, close to the active zone [49].

Several synaptic proteins are known to bind calcium. The integral synaptic vesicle protein, synaptotagmin, has been the candidate most intensively studied. The calcium binding sites of synaptotagmin reside in its two C2 domains, homologous to the calcium-binding region in protein kinase C. The low affinity binding of this domain for calcium is consistent with the concentrations of calcium needed to stimulate membrane fusion. Furthermore, various perturbation studies of synaptotagmin function, including protein overexpression and microinjection of antibodies and peptides, has revealed a role for this protein in synaptic transmission [58, 59]. Moreover, synaptotagmin is known to be able to oligomerize, bind to the tSNARE proteins, syntaxin and SNAP-25, as well as bind to phospholipids in a calcium-dependent fashion [51, 60, 61]. These data are consistent with a model whereby calcium binding by synaptotagmin at the active zone is involved in the facilitation of SNARE complex formation and fusion. However, some of the many mammalian isoforms of synaptotagmin do not bind calcium, and gene knock-out studies in *C. elegans*, *D. melanogaster*, and mice have produced ambiguous results concerning the role for synaptotagmin in calcium dependent synaptic vesicle exocytosis [62-67]. Therefore, the true identity of the synaptic calcium sensor remains obscure.

SYNAPTIC VESICLE RECYCLING

Even at low levels of stimulation, the complement of synaptic vesicles would rapidly become depleted if there were no compensatory mechanism for their replenishment. Given the presence of variable-

frequency stimulation, and the ability of the nervous system to quickly alter the flux of neuronal firing, the rapid regeneration of synaptic vesicles is crucial for the maintenance of productive neurotransmission. In addition, the other consequence of unopposed synaptic vesicle exocytosis would be the large accumulation of membrane at the synapse, with the resulting drastic disruption of synaptic architecture. This problem is elegantly solved through the local recycling of synaptic vesicles at the nerve terminal [25, 35]. Synaptic vesicle recycling at the nerve terminal can regenerate and maintain the synaptic vesicle pool, while at the same time, balance the total surface area of the presynaptic membrane through a cycle of exo- and endocytosis [68].

Indeed, exocytosis is tightly coupled to endocytosis in the synaptic vesicle cycle. The nature of the tight coupling is most likely through a combination of sensitivities to the same biochemical trigger, calcium, as well as through the detection, by the endocytic machinery, of synaptic vesicle components delivered to the presynaptic plasma membrane [68]. Experimental evidence supports both of these potential coupling mechanisms. Formation of endocytic complexes is facilitated through calcium-dependent dephosphorylation of several proteins in the endocytic pathway by the phosphatase, calcineurin [69, 70]. Furthermore, the putative exocytic calcium sensor, synaptotagmin, binds to the alpha-adaptin subunit of the clathrin adaptor AP-2, serving to potentially nucleate the formation of a clathrin-coated pit at sites where synaptotagmin has been delivered to the plasma membrane [71]. In addition, in the vertebrate fish, lamprey, artificial uncoupling of exocytosis from endocytosis resulted in the endocytosis of synaptic vesicles only until the vesicle pool had replenished, arguing for a biochemical stimulus present at the plasma membrane which was triggering the endocytic process, namely, the presence of synaptic vesicle proteins delivered to the plasma membrane by exocytosis [72]. There is evidence showing that the rate of endocytosis directly correlates with the rate of exocytosis, further demonstrating the intimate coupling of these two processes [73, 74]. Moreover, recent data have implicated phosphoinositide metabolism in the exo-endocytic cycle of vesicles recycling from the plasma membrane, suggesting lipid-mediated coupling of these two processes [75-79].

Using styryl dyes that fluoresce upon embedding into lipid bilayers, the kinetics of evoked synaptic vesicle recycling have provided new data demonstrating robust vesicle turnover [80-82]. These experiments reveal kinetics that are dependent on the intensity and duration of stimulation. Following a brief 10 Hz stimulation, the half-life of synaptic vesicle endocytosis is roughly twenty seconds in central synapses as measured by these methods [81, 83, 84]. However, different kinetics of uptake have been found under different stimulation protocols and in different synapses, reflecting a potential functional role for various recycling dynamics within the diversity of the nervous system [68, 73]. Based on the kinetics of styryl dye unloading after internalization, it has been determined that roughly another 30 seconds is required after endocytosis for subsequent availability of the nascent vesicle for another round of exocytosis, placing the total time of recycling at just under one minute [85, 86]. Thus, the recycling pathway appears most critically important for maintenance of the reserve pool of synaptic vesicles.

Interestingly, there is segregation at the nerve terminal with respect to regions of exocytosis and endocytosis of synaptic vesicles. While exocytosis is thought to occur principally at the active zone proper, where synaptic vesicles are seen to be closely apposed to the presynaptic plasma membrane by electron microscopy, the major endocytic activity primarily occurs at the outer boundaries of the active zone, in a loose halo around the synaptic vesicle cluster

[68, 72, 87, 88]. In fact, the periphery of the active zone is enriched in proteins involved in endocytosis [88, 89]. The mechanism for this functional segregation of exo-and endocytosis is not understood, although clearly there must be a role for protein-protein and protein-membrane interactions that favor this distribution. Conceptually, a separation of these two processes in space would in some ways prevent a functional competition unproductive for neurotransmission, and there may also be steric requirements for the separation of the different machineries required for exo- and endocytosis.

TWO PUTATIVE PATHWAYS FOR SYNAPTIC VESICLE RETRIEVAL

In what way does the local recycling of synaptic vesicles occur? In the early 1970s, electron microscopic studies of stimulated nerve terminals revealed clathrin-mediated endocytosis to be a major pathway of synaptic vesicle retrieval after exocytosis [35]. This highly specialized version of a general endocytic pathway is necessary for the maintenance of the synaptic vesicle pool, and perturbation of this system results in rapid depletion of synaptic vesicles at the nerve terminal [90-92].

The clathrin-mediated pathway for endocytosis of synaptic vesicles has been rigorously supported through experimentation using a variety of methods, and likely involves the direct formation of a synaptic vesicle from the plasma membrane (Figure 2) [16]. Biochemically, the brain is an abundant source of clathrin-coat proteins, derived from a coated vesicle fraction enriched in synaptic vesicle proteins [93]. Furthermore, by immunofluorescence, clathrin-coat proteins are highly concentrated in the nerve terminal, indicative of a major role for these proteins in a synaptic process [68]. Moreover, cellular manipulation experiments, through which the clathrin-mediated endocytic pathway was perturbed by either peptide or antibody microinjection, have clearly shown a major role for this pathway in synaptic vesicle recycling [91, 92, 94]. Some of the most striking examples of the importance of the clathrin-mediated recycling pathway are seen in the microinjection studies using the living giant reticulospinal synapse of the vertebrate fish, lamprey. In this preparation, disrupting proteins involved in clathrin-mediated synaptic vesicle recycling demonstrate a crucial role for this pathway in the maintenance of the synaptic vesicle pool, at either low or high levels of stimulation [91, 92, 94]. Such a disruption of clathrin-mediated endocytosis in the lamprey reticulospinal synapse results in near total depletion of the synaptic vesicle cluster, with many "trapped" intermediates of the clathrin-mediated endocytic pathway seen at the periphery of the active zone [91, 92, 94]. Furthermore, genetic studies in *D. melanogaster* and mice have further supported a major role for clathrin-mediated synaptic vesicle recycling, demonstrating both lethality and severe neurological dysfunction [75, 95-99].

An alternative pathway for synaptic vesicle recycling has also been proposed, independent of clathrin-mediated endocytosis. This hypothesis stemmed from electrophysiological studies of membrane capacitance, as well as electron micrographs showing synaptic vesicles in a state of partial fusion with the plasma membrane [100]. Known as "kiss and run," this hypothesis posits that the vesicle may, rather than completely collapsing into the presynaptic plasma membrane, only form a transient fusion pore which may then be resealed quickly to reform the synaptic vesicle [25, 101]. Opening of the fusion pore would be sufficient for release of neurotransmitter, without compromising the overall integrity of the vesicle. Advantages for this pathway would be potentially faster recycling kinetics derived from reversal of a partial fusion event, as well as circumventing the need to sort vesicle con-

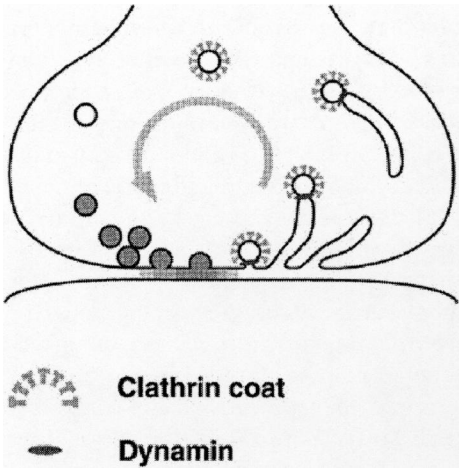

Figure 2. Synaptic vesicle recycling. Cartoon of synaptic vesicle recycling via the clathrin-mediated endocytic pathway. Synaptic vesicles, loaded with neurotransmitter, fuse with the presynaptic plasma membrane to release their neurotransmitter contents into the synaptic cleft, stimulating a postsynaptic response. After fusion and neurotransmitter release, synaptic vesicles are sorted and reformed by clathrin-mediated endocytosis, requiring the clathrin coat proteins and the dynamin GTPase (discussed below). Synaptic vesicle formation can occur directly from the plasma membrane, as well as from bulk internalized membranes after intense stimulation. Originally published in [16] and reproduced with permission from *Journal of Cell Biology*.

stituents from the pool of proteins and lipids present in the plasma membrane.

The most tenable evidence that the "kiss and run" mode of vesicle recycling occurs has been from capacitance studies of large secretory granule secretion [102-104]. The addition of membrane upon fusion of a large vesicle granule with the plasma membrane of a secretory cell is sufficient to detect an increase in the capacitance of the plasma membrane. Using these techniques, coupled with biochemical detectors of granule content release, it has been shown that a given granule may undergo a "flicker" state where the capacitance of the plasma membrane quickly changes in a reversible fashion in conjunction with secretion [101, 105, 106]. The amplitude of the capacitance change is similar to what would be seen with a single total fusion event. This led to the notion that the vesicle was reversibly fusing with the plasma membrane and releasing its contents in brief spurts.

Although the "flicker" or "kiss and run" mechanism of fusion has been demonstrated for secretion of these large vesicles, evidence for the same phenomenon occurring with synaptic vesicles has not been nearly as robust, particularly since the small size of synaptic vesicles is below the reliable detection limit for capacitance studies. Furthermore, the large secretory granules studied for capacitance changes are more akin to the large dense-core vesicles containing neuropeptides present in some neurons, rather than the small neurotransmitter-containing synaptic vesicles. These neuropeptide-containing large dense-core vesicles most likely do not undergo exocytosis at the active zone, and thus may have a qualitatively different mechanism of fusion distinct from that of synaptic vesicles [12]. This fact limits the ability to generalize a process involved with large granule secretion to synaptic vesicle exocytosis. The "kiss and run" model is still a viable one, however, and more experimentation may reveal a potential role for this pathway in synaptic vesicle recycling [73].

THE CAST OF PLAYERS IN SYNAPTIC VESICLE RETRIEVAL

Clathrin-mediated synaptic vesicle retrieval involves an intricate sequence of protein and membrane dynamics. A central question in this process is how the nascent vesicle bud is generated from the plasma membrane and then severed to form an independent organelle. Many proteins have been implicated in this process, from clathrin, to the adaptins, to a whole group of accessory proteins, the functions of

which have become increasingly studied in recent years [68, 107, 108].

Clathrin-coat proteins purified from the brain are comprised principally of clathrin-heavy and light chains, the hetero-tetrameric adaptor, AP2, and the large monomeric adaptor, AP180 [109]. Each of these proteins has independently been implicated as important components for clathrin-coat formation. The triskelion is the principal structural unit of clathrin assemblies [110]. The clathrin triskelion is comprised of three clathrin-heavy chains [111, 112]. The central hub of the triskelion is where the light chains are localized [113, 114], and the terminal domains represent the NH2-terminal regions of the clathrin-heavy chains which are known to be involved in protein-protein interactions with adaptors [115]. Rather than forming a major structural part of the triskelion, the clathrin-light chains are mainly thought to exert regulatory effects on coat formation [109, 116, 117]. The presence of clathrin-light chains inhibits clathrin cage formation at physiological pH, and phosphorylation of light chains may play a role in regulating the interaction of the light chains with the heavy chains [113, 118].

Clathrin triskelia can organize to form hexagonal and pentagonal lattices [109]. Geometric models of these shapes indicate that while triskelial hexagons can form flat sheets, triskelial pentagons introduce curvature in the lattice due to steric considerations of such a pentagonal assembly [119]. With the appropriate ratio of hexagons to pentagons, clathrin is able to form structures resembling geodesic domes, or "buckminster fullerenes" [120, 121]. Under acidic conditions below pH 6.5, clathrin itself can oligomerize into lattices and cages in solution comprised solely of triskelial units [111]; however, at physiologic pH, clathrin adaptor proteins are required for cage formation [122].

Both AP2 and AP180 can independently and synergistically stimulate free clathrin cage formation [123]. AP180 has also been shown, both *in vitro* and *in vivo* in *C. elegans* and *D. melanogaster*, to be necessary for the stringent size determination of the clathrin-coat for synaptic vesicles [96, 124, 125]. AP2 and AP180 are also able to bind to phospholipids [123, 126, 127] and therefore provide an additional structural link to the site of action for the clathrin-coat. In fact, purified clathrin-coat proteins can form coated buds on protein-free artificial liposomes, demonstrating that soluble components alone are sufficient to generate the structural intermediates in endocytosis [128]. In addition, the μ2-subunit of AP2 has been shown to bind to a tyrosine-based sorting motif present in synaptotagmin, and therefore, provides a link for the sorting of synaptic vesicle components implicit in clathrin-mediated synaptic vesicle retrieval [71,129]. Consistent with this idea, AP180 *C. elegans* mutants were shown to mislocalize the vSNARE, synaptobrevin, indicating an important role for AP180 in the sorting of this important exocytic protein [124].

Recently discovered in Drosophila, the novel synaptic protein, *stoned* B, has been shown to associate with clathrin-coat proteins and to be important in synaptic vesicle recycling [130-132]. *Stoned* B has structural homology to the μ2-subunit of AP2, and concordantly, interacts directly with synaptotagmin [133]. Deletion of the *stoned* B locus results in mislocalization of synaptotagmin and defects in neurotransmission attributed to a dysfunction of synaptic vesicle recyling [134-136]. This further demonstrates how proteins associated with the clathrin-coat can be intimately involved in sorting of synaptic vesicle proteins and in the efficiency of synaptic vesicle recycling.

RING AROUND THE COLLAR

In addition to the proteins comprising the clathrin-coat, several other proteins have been implicated in this endocytic pathway [68]. While clathrin-coat proteins

are involved in budding from the plasma membrane, they are not sufficient to cause fission of the bud into a vesicle. The first evidence that factors other than the clathrin-coat proteins were involved in clathrin-mediated synaptic vesicle retrieval came from the study of a Drosophila temperature-sensitive mutant severely defective in neurotransmission [90]. Morphological analysis of the *shibire* temperature sensitive mutation in *D. melanogaster* showed that, at the restrictive temperature, paralysis coincided with depletion of the synaptic vesicle cluster and the arrest of clathrin-mediated endocytosis at deeply invaginated buds (Figure 3) [90]. Interestingly, electron-dense collars were found at the necks of these clathrin-coated buds [90]. The mutant gene was subsequently found to encode the Drosophila ortholog of dynamin, a 100kDa GTPase initially cloned as a microtubule associated protein [137, 138]. Dynamin has three different isoforms totalling up to twenty-seven different splice variants [139]. Dynamin 1 is specific to the brain and is highly enriched at the nerve terminal. Dynamin 2 is ubiquitously expressed, while dynamin 3 is predominantly in testes, with some brain and lung expression.

Dynamin is comprised of five major domains: an NH2-terminal GTPase domain; a middle domain containing a region of coiled-coils potentially involved in dynamin-dynamin interactions; a pleckstrin-homology (PH) domain with affinities for phosphoinositides, and which may also participate in protein-protein interactions; a GTPase effector domain (GED) thought to stimulate dynamin GTPase activity as well as to regulate dynamin-dynamin oligomerization through two predicted coiled-coil segments; and a COOH-terminal region rich in prolines and arginines (PRD) known to bind several proteins containing src-homology 3 (SH3) domains (Figure 3C) [140]. The large NH2-terminal GTPase domain, the most highly conserved amongst the dynamins, is characterized by very high GTPase activity and low affinity for nucleotides. Indeed, these traits of a large GTPase with high hydrolytic activity and low nucleotide affinity have defined a family of GTPases, including members ranging in function from the immune system to mitochondrial dynamics [139, 141].

Two important lines of evidence initially implicated dynamin as an important endocytic protein in mammals. Overexpression, in mammalian cells, of a dynamin construct unable to bind GTP acted in a dominant negative fashion to potently block endocytosis [142, 143]. Furthermore, treatment of isolated synaptic membranes with brain cytosol and GTPγS, a non-hydrolyzable analog of GTP, could mimic the fission arrest phenomenon seen in the *shibire* mutant, with multiple electron dense rings constricting the membrane below a clathrin-coated bud (Figure 4) [15]. These electron-dense collars were immuno-gold positive for dynamin. Moreover, dynamin was shown to form stacks of rings in solution by electron microscopy [144], and purified dynamin alone deformed both natural membranes and artificial lipid bilayers into tubules with the approximate diameter of the neck of a clathrin-coated bud [128]. Upon addition of GTP, some tubules generated by dynamin on liposomes were seen to constrict in diameter, and many tubules fragmented to small vesicular structures [145]. Thus, fission of a nascent clathrin-coated bud from the plasma membrane requires dynamin, and specifically, a GTP-dependent function of dynamin. Given the *in vitro* and *in vivo* dynamics observed with dynamin and lipid bilayers, the GTP-dependent function is likely a mechano-chemical transduction of energy sufficient to mediate scission of the tubular neck of the clathrin-coated bud [146]. An alternative mechanism has also been proposed whereby dynamin may stimulate a downstream fission machinery in a GTP-dependent fashion [147], and it is possible that both mechanisms may work in tandem.

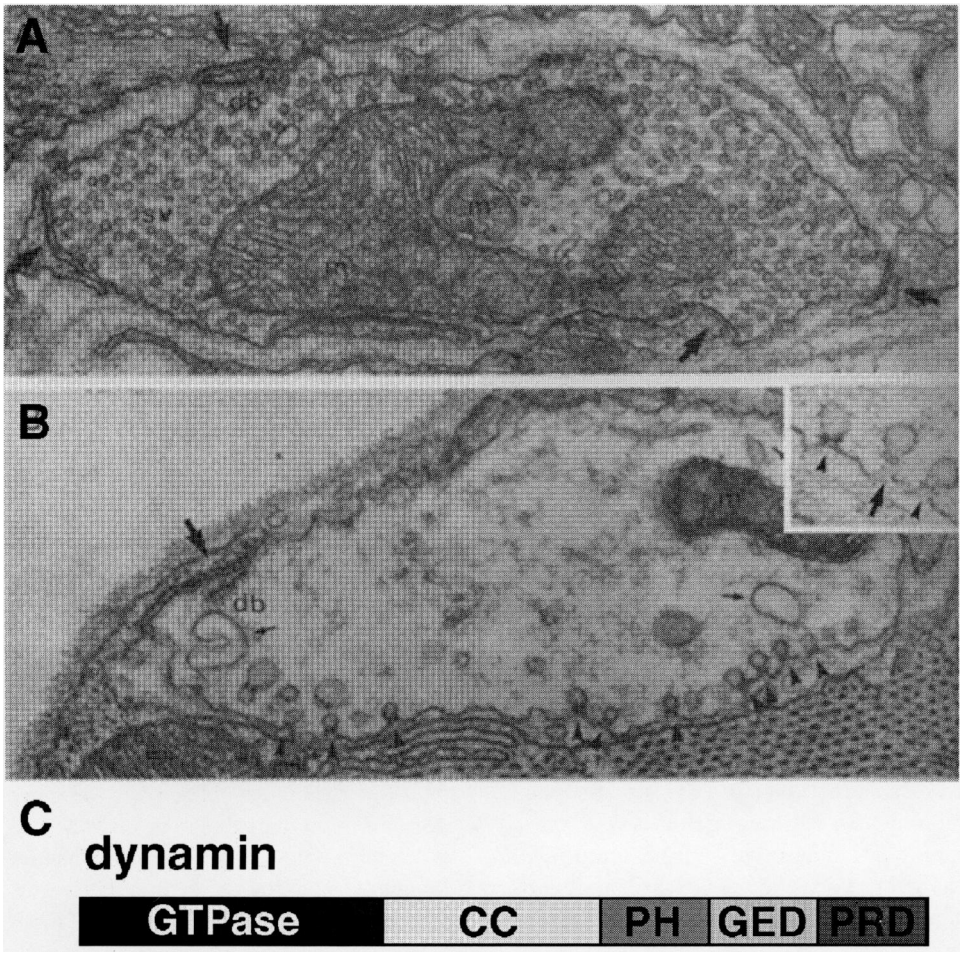

Figure 3. The shibire mutant in *D. melanogaster* displays a temperature-sensitive arrest of synaptic vesicle recycling due to failure of vesicle fission as a result of a defect in the gene encoding the GTPase, dynamin.

(**A**) At the permissive temperature, the pre-synaptic compartment is replete with synaptic vesicles, and the fly behaves normally.

(**B**) At the restrictive temperature, paralysis coincides with a depletion of synaptic vesicles, and an accumulation of clathrin-coated profiles arrested at a deeply invaginated state. Note the electron-dense ring-like structures evident at the neck of many of the coated buds. Originally published in [90] and reproduced with permission from the *Journal of Neuroscience*.

(**C**) The domain structure of dynamin, the 100 kDa GTPase product of the shibire gene. The GTPase activity of the NH2-terminal GTPase domain is required for fission of the nascent clathrin-coated bud into a vesicle. The COOH-terminal proline- and arginine-rich domain is responsible for dynamin binding to various SH3 domain-containing proteins. CC = coiled coils; GED = GTPase effector domain. Originally published in [172] and reproduced with permission from the author.

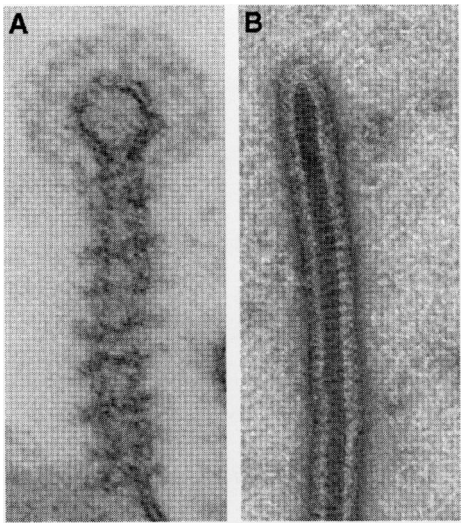

Figure 4. Membrane tubules generated by purified dynamin have a different protein coat compared with tubules generated by brain cytosol

(**A**) Thin section electron micrograph of epon-embedded membrane tubules generated in the presence of synaptic membranes, brain cytosol, and GTP_S. Note the presence of electron-dense rings similar to those seen in the shibire nerve terminals at the restrictive temperature. These tubules are highly immunoreactive against anti-dynamin antibodies.Originally published in [15] and reproduced with permission of *Nature*.

(**B**) Negative stain electron micrograph of membrane tubules generated in the presence of liposomes and purified rat brain dynamin. Note that although the dimensions of the membrane tubule are the same as compared with cytosol in A (roughly 25-100 nm diameter), the morphology of the protein coat on the tubule is different, with purified dynamin being represented by thinner rings spaced more closely together. This suggests that a protein complex is likely responsible for the thick electron-dense structure seen with brain cytosol. Originally published in [172] and reproduced by permission from the author.

Dynamin exists as dimers and tetramers in solution and is able to form high order oligomers under conditions of low salt, or on the surface of lipid bilayers. Recently, a 20 Ångstrom structure of dynamin oligomerized in a tubule has been solved by electron cryo-microscopy [148, 149]. This structure shows the unit of dynamin assembly to be a dimer, and a dynamin ring to be composed of dimeric oligomers with an eleven-fold axis of symmetry [148, 149]. Dynamin is assembled as a continuous stack of rings that is poised in such a way as to mediate constriction with conformational change [148, 149]. This model supports a mechano-chemical function for dynamin, although downstream effectors may still play a role in the fission reaction.

PROTEIN COMPLEXES IN THE ENDOCYTIC FISSION RING

Thin-section electron microscopic specimens of membrane tubules generated by purified dynamin do not exhibit the dense ring pattern observed when membranes are treated with brain cytosol and GTPγS. The rings generated by purified dynamin are only seen by negative stain electron microscopy, and they are thinner and spaced more closely together than the rings formed with brain cytosol (Figure 4) [150]. The rings seen with brain cytosol, therefore, most likely represent a complex of proteins involved in membrane deformation during clathrin-mediated endocytosis, which together are able to generate the electron density observed by thin sectioning.

The most likely candidates for the proteins involved in the endocytic fission ring include major dynamin interacting partners at the nerve terminal. One such protein is amphiphysin, a 128 kDa protein comprising a highly conserved NH2-terminal coiled-coil region and a COOH-terminal SH3 domain (Figure 5) [151]. The coiled-coil region is involved in protein-membrane interactions as well as dimerization/multimerization [152, 153], while the SH3 domain binds dynamin and the synap-

tic polyphosphoinositide phosphatase, synaptojanin [151, 154]. Furthermore, upstream of its SH3 domain, neuronal isoforms of amphiphysin contain partially overlapping sequences known to bind clathrin and AP2 [155], thus providing for a putative link between interactions with dynamin and the clathrin-coat [150].

The role of amphiphysin in endocytosis has been shown by the strong inhibition of endocytosis found using its SH3 domain for acute perturbation studies. Cells transiently expressing the amphiphysin SH3 domain exhibit a profound block in clathrin-mediated endocytosis [156]. Moreover, microinjection of the amphiphysin SH3 domain in the stimulated nerve terminal of the lamprey reticulospinal synapse results in depletion of synaptic vesicles secondary to a block in clathrin-mediated synaptic vesicle retrieval [91]. The block in synaptic vesicle recycling is at the late fission step, as numerous deeply invaginated clathrin-coated buds were seen accumulated at the periphery of the active zone [91]. Chronic perturbation of amphiphysin function mediated by targeted disruption of the amphiphysin gene leads to severe cognitive deficits correlated with dysfunctional synaptic vesicle recycling [99]. A subset of amphiphysin knockout mice also suffer premature death due to an enhanced susceptibility to seizures [99].

These data point to a role for amphiphysin in the high-efficiency synaptic vesicle recycling required for higher order brain functioning.

In vitro, amphiphysin forms a complex with dynamin, which, by comparison, forms more widely spaced electron dense rings visible also by thin sectioning [150]. These rings more closely resemble the morphology of the rings seen using total brain cytosol, rather than the rings seen with dynamin alone [150], supporting the notion that a complex of proteins is present at the thick electron-dense collar present at the tubular neck of the clathrin-coated bud. Moreover, recombinant amphiphysin alone was able to tubulate liposomes to roughly the same diameter as the tubules generated by dynamin [150]. Thus, the role of amphiphysin in the synaptic vesicle cycle may relate to its structural interaction with dynamin at the tubular neck of the clathrin-coated bud.

LIPID METABOLISM IN SYNAPTIC VESICLE RECYCLING

As mentioned, the other major binding partner of amphiphysin at the nerve terminal is the polyphosphoinositide phosphatase, synaptojanin (Figure 5) [154]. Synaptojanin is concentrated at the nerve terminal and is found on coated endocytic intermediates in an incubation using synaptic membranes with brain cytosol and GTPγS [157, 158]. This led to the speculation that phosphoinositide metabolism is an important part of the synaptic vesicle cycle [75].

Synaptojanin has two phosphoinositide phosphatase domains that mediate the dephosphorylation of the phosphoinositides, $PI_{4,5}P$ (PIP_2) and $PI_{3,4,5}P$ (PIP_3) [159]. The synaptojanin COOH-terminal proline-rich domain mediates protein-protein interactions. Both chronic and acute perturbations have implicated synaptojanin in synaptic vesicle recycling. Targeted disruption of the synaptojanin locus in mice leads to severe neurological deficits, failure to thrive, and a concomitant deficiency in neurotransmission [75]. An increase in brain PIP_2 levels compared with wild-type mice was noted, indicating a possible causal link between the phenotype and the defect in phosphoinositide metabolism [75]. Furthermore, microinjection studies in the lamprey synapse have demonstrated a block in endocytosis with the ensuing accumulation of clathrin-coated vesicles and the proliferation of an actin cytomatrix [92].

The importance of lipids in synaptic vesicle recycling has been underscored by several observations. Dynamin shows a preference for acidic phospholipids, with

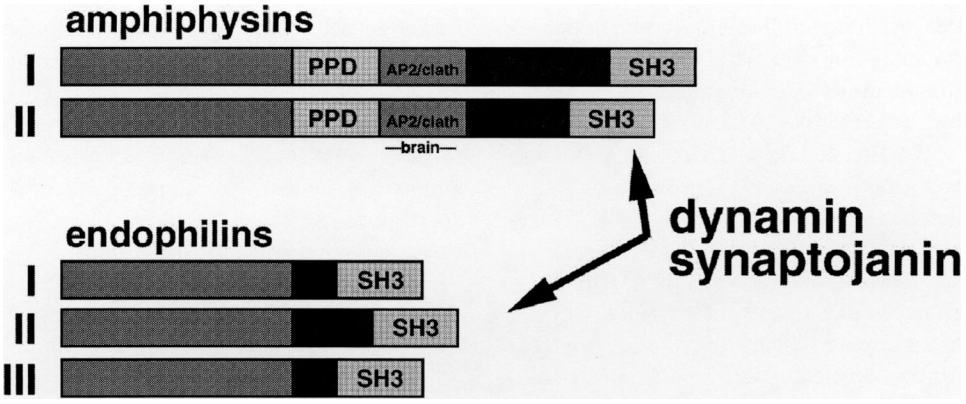

Figure 5. Major pre-synaptic dynamin binding partners. Amphiphysin and endophilin are proteins which are highly enriched at the synapse, and which interact with dynamin, and the polyphosotidlyinositol phosphatase, synaptojanin, via their COOH-terminal SH3 domains. Both proteins have a highly conserved NH2-terminal domain predicted to form alpha helices and coiled-coils. Brain isoforms of amphiphysin have a central domain with binding sites for clathrin and the clathrin adaptor protein, AP2. Originally published in [172] and reproduced by permission from the author.

its PH domain having an affinity for PIP_2 [160, 161]. PIP_2 is a biologically active lipid, and the observed effects of synaptojanin perturbation may reflect the affinity of the clathrin-coat adaptors to PIP_2, as well as the ability of PIP_2 to nucleate actin dynamics [76, 162]. Indeed, a brain-enriched isoform of a PI(4)P 5-kinase, which generates PIP_2 by the addition of a phosphate to position 5° of the inositol ring in the precursor, PI(4)P, has recently been identified [77]. This kinase is enriched at the nerve terminal and localizes to clathrin-coated intermediates in the cell-free incubation previously mentioned with brain cytosol [77].

Interestingly, amphiphysin has been reported to act as an inhibitor of phospholipase D, an enzyme mediating the conversion of phosphatidyl-choline to phosphatydic acid by cleavage of the choline moiety of the head group [163]. The action of phospholipase D is a part of an enzymatic cascade leading to PIP_2 generation, and therefore, inhibition of this enzyme would theoretically block this cascade, preventing additional formation of PIP_2. Along with its binding partner, synaptojanin, amphiphysin may work to decrease PIP_2 levels in a function that may have importance for synaptic vesicle dynamics. Thus, a putative cycle of phosphoinositide metabolism may occur in tandem with the synaptic vesicle cycle, whereby generation of PIP_2 after exocytosis leads to coated bud formation in addition to undefined actin dynamics, and removal of PIP_2 leads to vesicle uncoating and competence to enter the recycling pool of synaptic vesicles [76].

ENDOPHILIN AND SYNAPTIC VESICLE RECYCLING

Another major binding partner of dynamin and synaptojanin is endophilin 1, a 40kDa SH3 domain-containing protein enriched in the synapse (Figure 5) [154, 164]. Microinjection studies in the living giant reticulospinal synapse of lamprey have implicated endophilin in many stages of clathrin-mediated synaptic vesicle endocytosis, from early events generating deep membrane curvature in the developing clathrin-coated bud, to later stages such as fission and uncoating of the nascent vesi-

cle [92, 94]. Furthermore, endophilin was found to be necessary for generation of small synaptic-like microvesicles in a PC12 cell based assay [165].

In Drosophila, the endophilin 1 ortholog is essential, with mutants containing P-element insertions in the endophilin 1 gene leading to death at the third instar larval stage [97, 98]. Drosophila endophilin is a crucial part of synaptic function, and analysis of the nerve terminals in these mutants prior to death revealed depletion of synaptic vesicles and the presence of large vacuoles [97, 98, 166]. In some nerve terminals that were only mildly affected, numerous clathrin-coated intermediates were seen that remarkably resembled the shallow pits seen in the lamprey synapse upon microinjection of endophilin antibodies [94, 97].

In a cell-free assay with rat brain cytosol and synaptic membranes incubated with GTPγS, endophilin co-localized with dynamin on membrane tubules, and selective depletion of endophilin from the cytosol resulted in diminished numbers of dynamin-coated tubules [94]. These data suggest that endophilin plays a major role in synaptic vesicle recycling and that the generation or stabilization of the dynamin-coated membrane tubule at the neck of the clathrin-coated bud may be a part of its function at the synapse. Interestingly, both endophilin and amphiphysin, the two major presynaptic binding partners of dynamin, co-localize to the membrane tubules generated with brain cytosol [94, 150]. However, it is unclear whether these two SH3 domain-containing proteins are functioning in a similar manner with dynamin at the neck of the clathrin-coated bud, or whether they serve differential roles in space and/or time.

Recently, endophilin was biochemically identified in a cytosolic extract containing lysophosphatidic acid acyl transferase (LPAAT) activity, making endophilin the first cytosolic protein reported to have such an activity [165]. This acyl transferase activity, which generates phosphatidic acid (PA) by the transfer of arachidonoyl-CoA to lysophosphatidic acid (LPA), was thought to be crucial for the generation of membrane curvature by the conversion of an "inverse cone" lipid (LPA) to a "cone-shaped" shaped lipid (PA) in the cytoplasmic leaflet of the membrane [165]. The authors of this study speculated that this activity was necessary for the transition from the positive membrane curvature of the budding vesicle, to the negative membrane curvature of the neck of the clathrin-coated bud [165], thereby being involved in fission. No direct evidence for a membrane deforming activity of endophilin was shown, however.

Endophilin has since been shown to directly bind and deform lipid bilayers into tubular structures [167]. This property of endophilin is independent of lipid modifying enzymatic activity, thus, it remains unclear what role the reported LPAAT activity plays in induction of membrane curvature [167]. LPA is a relatively soluble lipid, and like most Type I lipids, it is likely not significantly present in biological membranes [168]. It has been suggested that perhaps endophilin undergoes the LPAAT reaction in the cytosol, with soluble LPA, and then partitions into the bilayer upon the creation of the insoluble reaction product, PA [32]. However, this would serve to promote positive rather than negative curvature. Moreover, at neutral biological pH, PA likely acts as a bilayer promoting lipid, rather than a Type II negative curvature promoting lipid-like PE [168]. Although the importance of the LPAAT activity of these proteins is as yet undefined, the ability of these proteins to bind certain lipids may prove important for their biological function.

An important aspect in membrane curvature formation by endophilin is the apparent requirement of an NH2-terminal amphipathic helix, which likely has properties enabling the protein to penetrate the lipid bilayer [167]. This amphipathic motif has also been noted in other proteins found to

drive membrane curvature. These proteins have been found associated with various cellular compartments, including amphiphysin 1 [167] and epsin [169] at the plasma membrane, an endophilin-like protein called endophilin B, localized to the Golgi complex and other intracellular membranes [167], muscle amphiphysin 2, localized to the muscle T-tubule system involved in coupling the excitation-contraction process [170], and RICH, a newly discovered family of proteins involved in actin dynamics [171]. The association of an amphipathic helix with the membrane may be a common mechanism whereby proteins can affect the process of membrane deformation via their partial insertion into the bilayer. Membrane active proteins such as these are likely central to the process of vesicle formation, enabling the efficient recycling of synaptic vesicles to allow for rapid and plastic neurotransmission.

CONCLUDING REMARKS

Fast point-to-point neurotransmission underlies the complex physiology of the nervous system. Elucidating the molecular mechanisms of this action is crucial to our understanding of nervous system function. The cell biology of the nerve terminal is an important piece in this puzzle, and within this context, the efficient recycling of synaptic vesicles serves to maintain the high transfer of information characteristic of neuronal activity. Undoubtedly, we have only scratched the surface of the complex molecular interactions that are a part of the synaptic vesicle recycling process, and much is left to be studied. Clearly, clathrin-mediated endocytosis is an important part of synaptic vesicle retrieval and remains the most well characterized pathway known to function in this process. The membrane recruitment of cytosolic proteins that are active in deforming the bilayer appears crucial to this process. It will be interesting to see whether parallel pathways exist which may define a unique biology within synaptic vesicle recycling. Studying the detailed function of the various macromolecules shown to play a role in clathrin-mediated synaptic vesicle endocytosis will provide us with more tools to model our understanding of the dynamic function of the nervous system.

REFERENCES:

1. Cowan, W.M. and Kandel, E.R. Brief history of synapses and synaptic transmission. In: Cowan, W.M., Sudhof, T.C., and Stevens, C.F., eds. *Synapses.* Johns Hopkins University Press; 2001: pp. 1-87.
2. Palay, S.L. and Palade, G. The fine structure of neurons. J. Biophys. Biochem. Cytol. 1:69-88, 1955.
3. De Robertis, E. and Bennett, H.S. Some features of the submicroscopic morphology of synapses in frog and earthworm. J. Biophys. Biochem. Cytol. 1:47-58, 1955.
4. Bennett, M.V. Gap junctions as electrical synapses. J. Neurocytology 26:349-366, 1997.
5. Unger, V.M., Kumar, N.M., Gilula, N.B., and Yeager, M. Three-dimensional structure of a recombinant gap junction membrane channel. Science 283:1176-1180, 1999.
6. Falk, M.M. Biosynthesis and structural composition of gap junction intercellular membrane channels. Eur. J. Cell Biol. 79:564-574, 2000.
7. Bennett, M.V. Physiology of electrotonic junctions. Ann. N.Y. Acad. Sci. 137:509-539, 1966.
8. Bennett, M.V. Electrical versus chemical neurotransmission. Res. Publ. Assoc. Res. Nerv. Ment. Dis. 50:58-90, 1972.
9. Kandel, E.R. Nerve Cells and behavior. In: Kandel, E.R., Schwarz, J.H., and Jessel, T.M., eds. *Principles of Neural Science,* fourth edition. New York: McGraw-Hill; 2000, pp. 19-35.
10. Gilliam, T.C., Kandel, E.R., Jessell, T.M. Genes and behavior. In: Kandel, E.R., Schwarz, J.H., and Jessel, T.M., eds. *Principles of Neural Science,* fourth edition. New York: McGraw-Hill; 2000, pp. 1414.
11. Heuser, J.E., and Reese, T.S. Structure of the synapse, Vol. 1, Part 1. Kandel, E.R., ed. Bethesda: American Physiological Society; 1977.
12. De Camilli, P., Haucke, V., Takei, K., and Mugnaini, E. The structure of synapses. In: Cowan, W.M., Sudhof, T.C., and Stevens, C.F., eds. *Synapses.* Baltimore: Johns Hopkins University Press; 2001: pp. 89-133.

13. Benson, D.L., Colman, D.R., and Huntley, G.W. Molecules, maps and synapse specificity. Nat. Rev. Neurosci. 2:899-909, 2001.
14. Gray, E.G. and Whittaker, V.P. The isolation of nerve endings from brain: an elctron microscopic study of cell fragments derived by homogenization and centrifugation. J. Anat. 96:79-88, 962.
15. Takei, K., McPherson, P.S., Schmid, S.L., De Camilli, P. Tubular membrane invaginations coated by dynamin rings are induced by GTPγS in nerve terminals. Nature 374:186-190, 1995.
16. Takei, K., Mundigl, O., Daniell, L., De Camilli, P. The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin. J. Cell Biol. 133:1237-1250, 1996.
17. Hannah, M.J., Schmidt, A.A., and Huttner, W.B. Synaptic vesicle biogenesis. Annu. Rev. Cell Dev. Biol. 15:733-798, 1999.
18. Jahn, R., Hell, J., and Maycox, P.R.W. Synaptic vesicles: key organelles involved in neurotransmission. J. Physiologie 84:128-133, 1990.
19. Couteauxd R.F and Pecot-Dechavassined, M. Synaptic vesicles and pouches at the level of "active zones" of the neuromuscular junction. C.R. Hebd. Seances Acad. Sci. D. Sci. Nat. 217:2346-2349, 1970.
20. Fatt, P. and Katz, B. An analysis of the end-plate potential recorded with an intracellular electrode. J. Physiol. 115:320-370, 1951.
21. Fatt, P. and Katz, B. Spontaneous subthreshold activity at motor nerve endings. J. Physiol. 117:109-128, 1952.
22. Del Castillo, J. and Katz, B. Quantal components of the end-plate potential. J. Physiol. 124:560-573, 1954.
23. Katz, B. and Miledi, R. The quantal release of transmitter substances. Curtis, D.R. and McIntyre, A.K., eds.. New York: Springer-Verlag; 1965.
24. Kuffler, S.W. and Yoshikami, D. The number of transmitter molecules in a quantum: an estimate from iontophoretic application of acetylcholine at the neuromuscular synapse. J. Physiol. 251:465-482, 1975.
25. Ceccarelli, B., Hurlbut, W.P., and Mauro, A. Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. J. Cell Biol. 57:499-524, 1973.
26. Heuser, J.E., Reese, T.S., Dennis, M.J., Jan, Y., Jan, L., and Evans, L. Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. J. Cell Biol. 81:275-300, 1979.
27. Haimann, C., Torri-Tarelli, F., Fesce, R., and Ceccarelli, B. Measurement of quantal secretion induced by ouabain and its correlation with depletion of synaptic vesicles. J. Cell Biol. 101:1953-1965, 1985.
28. Heuser, J. The role of coated vesicles in recycling of synaptic vesicle membrane. Cell Biol. Int. Rep. 13:1063-1076, 1989.
29. Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M.A., Keen, J.H., and McGraw, T.E. Acute cholesterol depletion inhibits clathrin-coated pit budding. Proc. Natl. Acad. Sci. U.S.A. 96:6775-6780, 1999.
30. Rodal, S.K., Skretting, G., Garred, O., Vilhardt, F., van Deurs, B., and Sandvig, K. Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. Mol. Biol. Cell 10:961-974, 1999.
31. Thiele, C., Hannah, M.J., Fahrenholz, F., and Huttner, W.B. Cholesterol binds to synaptophysin and is required for biogenesis of synaptic vesicles. Nat. Cell. Biol. 2:42-49, 2000. java/Propub/cellbio/ncb0100_0142.fulltext java/Propub/cellbio/ncb0100_0142.abstract.
32. Huttner, W.B. and Schmidt, A. Lipids, lipid modification and lipid-protein interaction in membrane budding and fission — insights from the roles of endophilin A1 and synaptophysin in synaptic vesicle endocytosis. Curr. Opin. Neurobiol. 10:543-551, 2000.
33. Tsukita, S. and Ishikawa, H. The movement of membranous organelles in axons. Electron microscopic identification of anterogradely and retrogradely transported organelles. J. Cell Biol. 84:513-530, 1980.
34. Okada, Y., Yamazaki, H., Sekine-Aizawa, Y., and Hirokawa N. The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. Cell 81:769-780, 1995.
35. Heuser, J.E. and Reese, T.S. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J. Cell Biol. 57:315-344, 1973.
36. Regehr, W.G. and Stevens, C.F. Physiology of synaptic transmission and short-term plasticity. In: Cowan, W.M., Sudhof, T.C., and Stevens, C.F., eds. *Synapses.* Baltimore, Maryland: Johns Hopkins University Press; 2001, pp. 135-175.
37. Elmqvist, D. and Quastel, D.M. A quantitative study of end-plate potentials in isolated human muscle. J. Physiol. 178:505-529, 1965.
38. Liu, G. and Tsien, R.W. Properties of synaptic transmission at single hippocampal synaptic boutons. Nature 375:404-408, 1995.
39. Rosenmund, C. and Stevens, C.F. Definition of the readily releasable pool of vesi-

cles at hippocampal synapses. Neuron 16:1197-1207, 1996.
40. Stevens, C.F. and Tsujimoto, T. Estimates for the pool size of releasable quanta at a single central synapse and for the time required to refill the pool. Proc. Natl. Acad. Sci. U.S.A. 92:846-849, 1995.
41. Zucker, R.S. Changes in the statistics of transmitter release during facilitation. J. Physiol. 229:787-810, 1973.
42. Schikorski, T. and Stevens, C.F. Quantitative ultrastructural analysis of hippocampal excitatory synapses. J. Neurosci. 17:5858-5867, 1997.
43. De Camilli, P., Harris, S.M., Jr., Huttner, W.B., and Greengard. P. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. J. Cell Biol. 96:1355-1373, 1983b.
44. De Camilli, P., Cameron, R., and Greengard, P. Synapsin I (protein I) a nerve terminal specific phosphoprotein: its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. J. Cell Biol. 96:1337-1354, 1983a.
45. Huttner, W.B., Schiebler, W., Greengard, P., and De Camilli, P. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. J. Cell Biol. 96:1374-1388, 1983.
46. Bähler, M., Benfenati, F., Valtorta, F., and Greengard, P. The synapsins and the regulation of synaptic function. Bioessays 12:259-263, 1990.
47. De Camilli, P., Benfenati, F., Valtorta, F., and Greengard, P. The synapsins. Annu. Rev. Cell Biol. 6:433-460, 1990.
48. Chi, P., Greengard, P., and Ryan, T.A. Synapsin dispersion and reclustering during synaptic activity. Nat. Neurosci. 4:1187-1193, 2001.
49. Lin, R.C. and Scheller, R.H. Mechanisms of synaptic vesicle exocytosis. Annu. Rev Cell Dev Biol 16:19-49, 2000.
50. Rothman, J.E. Mechanisms of intracellular protein transport. Nature 372:55-63, 1994.
51. Schiavo, G., Stenbeck, G., Rothman, J.E., and Söllner, T.H. Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. Proc. Natl. Acad. Sci. U.S.A. 94:997-1001, 1997.
52. Brunger, A.T. Structure of proteins involved in synaptic vesicle fusion in neurons. Annu. Rev. Biophys. Biomol. Struct. 30:157-171, 2001.
53. Parlati, F., McNew, J.A., Fukuda, R., Miller, R., Sollner, T.H., and Rothman, J.E. Topological restriction of SNARE-dependent membrane fusion. Nature 407:194-198, 2000.
54. McNew, J.A., Parlati, F., Fukuda, R., Johnston, R.J., Paz, K., Paumet, F., Sollner, T.H., Rothman, J.E. Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. Nature 407:153-159, 2000.
55. Pellizzari, R., Rossetto, O., Schiavo, G., and Montecucco, C. Tetanus and botulinum neurotoxins: mechanism of action and therapeutic uses. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 354:259-268, 1999.
56. Hunt, J.M., Bommert, K., Charlton, M.P., Kistner, A., Habermann, E., Augustine, G.J., and Betz, H. A post-docking role for synaptobrevin in synaptic vesicle fusion. Neuron 12:1269-1279, 1994.
57. Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T.H., and Rothman, J.E. SNAREpins: minimal machinery for membrane fusion. Cell 92:759-772, 1998.
58. Bommert, K., Charlton, M.P., DeBello, W.M., Chin, G.J., Betz, H., and Augustine, G.J. Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. Nature 363:163-165, 1993.
59. Mikoshiba, K., Fukuda, M., Moreira, J.E., Lewis, F.M., Sugimori, M., Niinobe, M., and Llinas, R. Role of the C2A domain of synaptotagmin in transmitter release as determined by specific antibody injection into the squid giant synapse preterminal. Proc. Natl. Acad. Sci. U.S.A. 92:10703-10707, 1995.
60. Chapman, E.R., Hanson, P.I., An, S., and Jahn, R. Ca2+ regulates the interaction between synaptotagmin and syntaxin 1. J. Biol. Chem. 270:23667-23671, 1995.
61. Li, C., Ullrich, B., Zhang, J.Z., Anderson, R.G.W., Brose, N., Südhof, T.C. Ca2+-dependent and -independent activities of neural and non-neural synaptotagmins. Nature 375:594-599, 1995.
62. Nonet, M.L., Grundahl, K., Meyer, B.J., and Rand, J.B. Synaptic function is impaired but not eliminated in *C. elegans* mutants lacking synaptotagmin. Cell 73:1291-1305, 1993.
63. Littleton, J.T., Stern, M., Schulze, K., Perin, M., and Bellen, H.J. Mutational analysis of Drosophila synaptotagmin demonstrates its essential role in Ca(2+)-activated neurotransmitter release. Cell 74:1125-1134, 1993.
64. Broadie, K., Bellen, H.J., DiAntonio, A., Littleton, J.T., andn Schwarz, T.L. Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic

transmission. Proc. Natl. Acad. Sci. U.S.A. 91:10727-10731, 1994.
65. Littleton, J.T., Stern, M., Perin, M., and Bellen, H.J. Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in Drosophila synaptotagmin mutants. Proc. Natl. Acad. Sci. U.S.A. 91:10888-10892, 1994.
66. Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F., and Südhof, T.C. Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse. 79:717-727, 1994.
67. Chapman, E.R. Synaptotagmin: a Ca(2+) sensor that triggers exocytosis? Nat. Rev. Mol. Cell Biol. 3:498-508, 2002.
68. De Camilli, P., Slepnev, V.I., Shupliakov, O., and Brodin, L. Synaptic vesicle endocytosis. In: Cowan, W.M., Sudhof, T.C., and Stevens, C.F., eds. *Synapses.* Baltimore: Johns Hopkins University Press; 2001, pp. 217-274.
69. Robinson, P.J., Liu, J.P., Powell, K.A., Fykse, E.M., and Südhof, T.C. Phosphorylation of dynamin I and synaptic-vesicle recycling. Trends Neurosci. 17:348-353,1994.
70. Slepnev, V.I., Ochoa, G.C., Butler, M.H., Grabs, D., and Camilli, P.D. Role of phosphorylation in regulation of the assembly of endocytic coat complexes. Science 281:821-824, 1998.
71. Haucke, V. and De Camilli, P. AP-2 recruitment to synaptotagmin stimulated by tyrosine-based endocytic motifs. Science 285:1268-1271, 1999.
72. Gad, H., Low, P., Zotova, E., Brodin, L., and Shupliakov, O. Dissociation between Ca2+-triggered synaptic vesicle exocytosis and clathrin-mediated endocytosis at a central synapse. Neuron 21:607-616, 1998.
73. Klingauf, J., Kavalali, E.T., and Tsien, R.W. Kinetics and regulation of fast endocytosis at hippocampal synapses. Nature 394:581-585, 1998.
74. Marks, B. and McMahon, H.T. Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals. Curr. Biol. 8:740-749, 1998.
75. Cremona, O., Di Paolo, G., Wenk, M.R., Luthi, A., Kim, W.T., Takei, K., Daniell, L., Nemoto, Y., Shears, S.B., Flavell, R.A. Essential role of phosphoinositide metabolism in synaptic vesicle recycling. Cell 99:179-188, 1999.
76. Cremona, O. and De Camilli, P. Phosphoinositides in membrane traffic at the synapse. J. Cell Sci. 114:1041-1052, 2001.
77. Wenk, M.R., Pellegrini, L., Klenchin, V.A., Di Paolo, G., Chang, S., Daniell, L., Arioka, M., Martin, T.F., and De Camilli, P. PIP kinase Igamma is the major PI(4,5)P(2) synthesizing enzyme at the synapse. Neuron 32:79-88, 2001.
78. Kim, W.T., Chang, S., Daniell, L., Cremona, O., Di Paolo, G., and De Camilli, P. Delayed reentry of recycling vesicles into the fusion-competent synaptic vesicle pool in synaptojanin 1 knockout mice. Proc. Natl. Acad. Sci. U.S.A. 99:17143-17148, 2002.
79. Czech, M.P. Dynamics of phosphoinositides in membrane retrieval and insertion. Annu. Rev. Physiol. 65:791-815, 2003.
80. Betz, W.J., Mao, F., and Bewick, G.S. Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. J. Neurosci. 12:363-375,1992.
81. Ryan, T.A. and Smith, S.J. Vesicle pool mobilization during action potential firing at hippocampal synapses. Neuron 14:983-989, 1995.
82. Betz, W.J., Mao, F., and Smith, C.B. Imaging exocytosis and endocytosis. Curr. Opin. Neurobiol. 6:365-371, 1996.
83. Wu, L.G. and Betz, W.J. Nerve activity but not intracellular calcium determines the time course of endocytosis at the frog neuromuscular junction. Neuron 17:769-779, 1996.
84. Ryan, T.A., Smith, S.J., and Reuter, H. The timing of synaptic vesicle endocytosis. Proc. Natl. Acad. Sci. U.S.A. 93:5567-5571, 1996.
85. Betz, W.J. and Bewick, G.S. Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. Science 255:200-203, 1992.
86. Ryan, T.A., Reuter, H., Wendland, B., Schweizer, F.E., Tsien, R.W., and Smith, S.J. The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. Neuron 11:713-724, 1993.
87. Teng, H. and Wilkinson, R.S. Clathrin-mediated endocytosis near active zones in snake motor boutons. J. Neurosci. 20:7986-7993, 2000.
88. Jarousse, N. and Kelly, R.B. Endocytotic mechanisms in synapses. Curr. Opin. Cell Biol. 13:461-469, 2001.
89. Roos, J. and Kelly, R.B. The endocytic machinery in nerve terminals surrounds sites of exocytosis. Curr. Biol. 9:1411-1414, 1999.
90. Koenig, J.H. and Ikeda, K. Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval. J. Neurosci. 9:3844-3860, 1989.
91. Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., De

Camilli, P., and Brodin, L. Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions. Science 276:259-263, 1997.
92. Gad, H., Ringstad, N., Low, P., Kjaerulff, O., Gustafsson, J., Wenk, M., Di Paolo, G., Nemoto, Y., Crun, J., Ellisman, M.H., De Camilli. P., Shupliakov, O., and Brodin, L. Fission and uncoating of synaptic clathrin-coated vesicles are perturbed by disruption of interactions with the SH3 domain of endophilin. Neuron 27:301-312, 2000.
93. Maycox, P.R., Link, E., Reetz, A., Morris, S.A., and Jahn, R. Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling. J. Cell Biol. 118:1379-1388, 1992.
94. Ringstad, N., Gad, H., Low, P., Di Paolo, G., Brodin, L., Shupliakov, O., and De Camilli, P. Endophilin/SH3p4 is required for the transition from early to late stages in clathrin-mediated synaptic vesicle endocytosis. Neuron 24:143-154, 1999.
95. Gonzalez-Gaitan, M. and Jackle, H. Role of Drosophila alpha-adaptin in presynaptic vesicle recycling. Cell 88:767-776, 1997.
96. Zhang, B., Koh, Y.H., Beckstead, R.B., Budnik, V., Ganetzky, B., and Bellen, H.J. Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. Neuron 21:1465-1475, 1998.
97. Guichet, A., Wucherpfennig, T., Dudu, V., Etter, S., Wilsch-Brauniger, M., Hellwig, A., Gonzalez-Gaitan, M., Huttner, W.B., and Schmidt, A.A. Essential role of endophilin A in synaptic vesicle budding at the Drosophila neuromuscular junction. Embo. J. 21:1661-1672, 2002.
98. Verstreken, P., Kjaerulff, O., Lloyd, T.E., Atkinson, R., Zhou, Y., Meinertzhagen, I.A., and Bellen, H.J. Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release. Cell 109:101-112, 2002.
99. Di Paolo, G., Sankaranarayanan, S., Wenk, M.R., Daniell, L., Perucco, E., Caldarone, B.J., Flavell, R., Picciotto, M.R., Ryan, T.A., Cremona, O., and De Camilli, P. Decreased synaptic vesicle recycling efficiency and cognitive deficits in amphiphysin 1 knockout mice. Neuron 33:789-804, 2002.
100. Koenig, J.H. and Ikeda, K. Synaptic vesicles have two distinct recycling pathways. J. Cell Biol. 135:797-808, 1996.
101. Fesce, R., Grohovaz, F., Valtorta, F., and Meldolesi, J. Neurotransmitter release: Fusion or "kiss-and-run"? Trends Cell Biol. 4:1-4, 1994.
102. Breckenridge, L.J. and Almers, W. Currents through the fusion pore that forms during exocytosis of a secretory vesicle. Nature 328:814-817, 1987.
103. Breckenridge, L.J. and Almers, W. Final steps in exocytosis observed in a cell with giant secretory granules. Proc. Natl. Acad. Sci. U.S.A. 84:1945-1949, 1987.
104. Spruce, A.E., Breckenridge, L.J., Lee, A.K., and Almers, W. Properties of the fusion pore that forms during exocytosis of a mast cell secretory vesicle. Neuron 4:643-654, 1990.
105. Almers, W. and Tse, F.W. Transmitter release from synapses: does a preassembled fusion pore initiate exocytosis? Neuron 4:813-818, 1990.
106. Neher, E. Secretion without full fusion. Nature 363:497-498, 1993.
107. Schmid, S.L. Clathrin-coated vesicle formation and protein sorting: an integrated process. Annu. Rev. Biochem. 66:511-548, 1997.
108. Slepnev, V.I. and De Camilli, P. Accessory factors in clathrin-dependent synaptic vesicle endocytosis. Nat. Rev. Neurosci. 1:161-172, 2000.
109. Brodsky, F.M., Chen, C.Y., Knuehl, C., Towler, M.C., and Wakeham, D.E. Biological basket weaving: formation and function of clathrin-coated vesicles. Annu. Rev. Cell Dev. Biol. 17:517-568, 2001.
110. Brodsky, F.M. Clathrin structure characterized with monoclonal antibodies. II. Identification of *in vivo* forms of clathrin. J. Cell Biol. 101:2055-2062, 1985.
111. Brodsky, F.M. Living with clathrin: its role in intracellular membrane traffic. Science 242:1396-1402, 1988.
112. Nathke, I.S., Heuser, J., Lupas, A., Stock, J., Turck, C.W., and Brodsky, F.M. Folding and trimerization of clathrin subunits at the triskelion hub. Cell, 68:899-910, 1992.
113. Brodsky, F.M., Hill, B.L., Acton, S.L., Nathke, I., Wong, D.H., Ponnambalam, S., and Parham, P. Clathrin light chains: arrays of protein motifs that regulate coated-vesicle dynamics. Trends Biochem. Sci. 16:208-213, 1991.
114. Liu, S.H., Wong, M.L., Craik, C.S., and Brodsky, F.M. Regulation of clathrin assembly and trimerization defined using recombinant triskelion hubs. Cell 83:257-267, 1995.
115. ter Haar, E., Harrison, S.C., and Kirchhausen, T. Peptide-in-groove interactions link target proteins to the beta- propeller of clathrin. Proc. Natl. Acad. Sci. U.S.A. 97:1096-1100, 2000.
116. Winkler, F.K. and Stanley, K.K. Clathrin heavy chain, light chain interactions. Embo. J. 2:1393-1400, 1983.
117. Ybe, J.A., Greene, B., Liu, S.H., Pley, U., Parham, P., and Brodsky, F.M. Clathrin

self-assembly is regulated by three light-chain residues controlling the formation of critical salt bridges. Embo. J. 17:1297-1303, 1998.
118. Chu, D.S., Pishvaee, B., and Payne, G.S. A modulatory role for clathrin light chain phosphorylation in Golgi membrane protein localization during vegetative growth and during the mating response of Saccharomyces cerevisiae. Mol. Biol. Cell 10:713-726, 1999.
119. Kirchhausen, T. Clathrin. Annu. Rev. Biochem. 69:699-727, 2000.
120. Smith, C.J., Grigorieff, N., and Pearse, B.M. Clathrin coats at 21 A resolution: a cellular assembly designed to recycle multiple membrane receptors. Embo. J. 17:4943-4953, 1998.
121. Musacchio, A., Smith, C.J., Roseman, A.M., Harrison, S.C., Kirchhausen, T., and Pearse, B.M. Functional organization of clathrin in coats: combining electron cryomicroscopy and X-ray crystallography. Mol. Cell 3:761-770, 1999.
122. Ahle, S. and Ungewickell, E. Purification and properties of a new clathrin assembly protein. Embo. J. 5:3143-3149, 1986.
123. Ford, M.G., Pearse, B.M., Higgins, M.K., Vallis, Y., Owen, D.J., Gibson, A., Hopkins, C.R., Evans, P.R., and McMahon, H.T. Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. Science 291:1051-1055, 2001.
124. Nonet, M.L., Holgado, A.M., Brewer, F., Serpe, C.J., Norbeck, B.A., Holleran, J., Wei, L., Hartwieg, E., Jorgensen, E.M., and Alfonso, A. UNC-11, a Caenorhabditis elegans AP180 homologue, regulates the size and protein composition of synaptic vesicles. Mol. Biol. Cell. 1999, 10:2343-2360, 1999.
125. McMahon, H.T. Endocytosis: an assembly protein for clathrin cages. Curr. Biol. 9:R332-335, 1999.
126. De Camilli, P. and Takei, K. Molecular mechanisms in synaptic vesicle endocytosis and recycling. Neuron 16:481-486, 1996.
127. Mao, Y., Chen, J., Maynard, J.A., Zhang, B., and Quiocho, F.A. A novel all helix fold of the AP180 amino-terminal domain for phosphoinositide binding and clathrin assembly in synaptic vesicle endocytosis. Cell 104:433-440, 2001.
128. Takei, K., Haucke, V., Slepnev, V., Farsad, K., Salazar, M., Chen, H., and De Camilli, P. Generation of coated intermediates of clathrin-mediated endocytosis on protein-free liposomes. Cell 94:131-141, 1998.
129. Haucke, V., Wenk, M.R., Chapman, E.R., Farsad, K., and De Camilli, P. Dual interaction of synaptotagmin with μ2- and alpha-adaptin facilitates clathrin-coated pit nucleation. Embo. J. 19:6011-6019, 2000.
130. Andrews, J., Smith, M., Merakovsky, J., Coulson, M., Hannan, F., and Kelly, L.E. The *stoned* locus of Drosophila melanogaster produces a dicistronic transcript and encodes two distinct polypeptides. Genetics 143:1699-1711, 1996.
131. Martina, J.A., Bonangelino, C.J., Aguilar, R.C., and Bonifacino, J.S. Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery. J. Cell Biol. 153:1111-1120, 2001.
132. Walther, K., Krauss, M., Diril, M.K., Lemke, S., Ricotta, D., Honing, S., Kaiser, S., Haucke, and V. Human *stoned* B interacts with AP-2 and synaptotagmin and facilitates clathrin-coated vesicle uncoating. Embo. Rep. 2:634-640, 2001.
133. Phillips, A.M., Smith, M., Ramaswami, M., and Kelly, L.E. The products of the Drosophila *stoned* locus interact with synaptic vesicles via synaptotagmin. J. Neurosci. 20:8254-8261, 2000.
134. Fergestad, T., Davis, W.S., and Broadie, K. The *stoned* proteins regulate synaptic vesicle recycling in the presynaptic terminal. J. Neurosci. 19:5847-5860, 1999.
135. Fergestad, T. and Broadie, K. Interaction of *stoned* and synaptotagmin in synaptic vesicle endocytosis. J. Neurosci. 21:1218-1227, 2001.
136. Stimson, D.T., Estes, P.S., Rao, S., Krishnan, K.S., Kelly, L.E., and Ramaswami, M. Drosophila *stoned* proteins regulate the rate and fidelity of synaptic vesicle internalization. J. Neurosci. 21:3034-3044, 2001.
137. Shpetner, H.S. and Vallee, R.B. Identification of dynamin, a novel mechanochemical enzyme that mediates interactions between microtubules. Cell 59:421-432, 1989.
138. van der Bliek, A.M. and Meyerowitz, E.M. Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. Nature 351:411-414, 1991.
139. McNiven, M.A., Cao, H., Pitts, K.R., and Yoon, Y. The dynamin family of mechanoenzymes: pinching in new places. Trends Biochem. Sci. 25:115-120, 2000.
140. Hinshaw, J.E. Dynamin and its role in membrane fission. Annu. Rev. Cell Dev. Biol. 16:483-519, 2000.
141. Danino, D. and Hinshaw, J.E. Dynamin family of mechanoenzymes. Curr. Opin. Cell Biol. 13:454-460, 2001.
142. van der Bliek, A.M., Redelmeier, T.E., Damke, H., Tisdale, E.J., Meyerowitz,

E.M., and Schmid, S.L. Mutations in human dynamin block an intermediate stage in coated vesicle formation. J. Cell Biol. 122:553-563, 1993.

143. Damke, H., Baba, T., Warnock, D.E., and Schmid, S.L. Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. J. Cell Biol. 127:915-934, 1994.
144. Hinshaw, J.E. and Schmid, S.L. Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. Nature 374:190-192, 1995.
145. Sweitzer, S.M. and Hinshaw, J.E. Dynamin undergoes a GTP-dependent conformational change causing vesiculation. Cell 93:1021-1029, 1998.
146. Marks, B., Stowell, M.H., Vallis, Y., Mills, I.G., Gibson, A., Hopkins, C.R., and McMahon, H.T. GTPase activity of dynamin and resulting conformation change are essential for endocytosis. Nature 410:231-235, 2001.
147. Sever, S., Muhlberg, A.B., and Schmid, S.L. Impairment of dynamin's GAP domain stimulates receptor-mediated endocytosis. Nature 398:481-486, 1999.
148. Zhang, P. and Hinshaw, J.E. Three-dimensional reconstruction of dynamin in the constricted state. Nat. Cell Biol. 3:922-926, 2001.
149. Klockow, B., Tichelaar, W., Madden, D.R., Niemann, H.H., Akiba, T., Hirose, K., and Manstein, D.J. The dynamin A ring complex: molecular organization and nucleotide-dependent conformational changes. Embo. J. 21:240-250, 2002.
150. Takei, K., Slepnev, V.I., Haucke, V., and De Camilli, P. Functional partnership between amphiphysin and dynamin in clathrin- mediated endocytosis. Nat. Cell Biol. 1:33-39, 1999.
151. David, C., McPherson, P.S., Mundigl, O., and De Camilli, P.A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. Proc. Natl. Acad. Sci. U.S.A. 93:331-335, 1996.
152. Wigge, P., Kohler, K., Vallis, Y., Doyle, C.A., Owen, D., Hunt, S.P., and McMahon, H.T. Amphiphysin heterodimers: potential role in clathrin-mediated endocytosis. Mol. Biol. Cell 8:2003-2015, 1997.
153. Ramjaun, A.R., Micheva, K.D, Bouchelet, I., and McPherson, P.S. Identification and characterization of a nerve terminal-enriched amphiphysin isoform. J. Biol. Chem. 272:16700-16706, 1997.
154. de Heuvel, E., Bell, A.W., Ramjaun, A.R., Wong, K., Sossin, W.S., and McPherson, P.S. Identification of the major synaptojanin-binding proteins in brain. J. Biol. Chem. 272:8710-8716, 1997.
155. Slepnev, V.I., Ochoa, G.C., Butler, M.H., and De Camilli, P. Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1, and disruption of clathrin coat function mediated by amphiphysin fragments comprising these sites. J. Biol. Chem. 275:17583-17589 2000.
156. Wigge, P., Vallis, Y., and McMahon, H.T. Inhibition of receptor-mediated endocytosis by the amphiphysin SH3 domain. Curr. Biol. 1997, 7:554-560.
157. Haffner, C., Takei, K., Butler, M., David, C., Hudson, A., Grabs, D., and De Camilli, P. Evolutionary conservation of synaptojanin, an inositol 5-phopshatase highly concentrated at clathrin coats in nerve terminals. Mol. Biol. Cell 7 Suppl.:78a, 1996.
158. Haffner, C., Takei, K., Chen, H., Ringstad, N., Hudson, A., Butler, M.H., Salcini, A.E., Di Fiore, P.P., and De Camilli, P. Synaptojanin 1: localization on coated endocytic intermediates in nerve terminals and interaction of its 170 kDa isoform with Eps15. FEBS Lett 419:175-180, 1997.
159. McPherson, P.S., Garcia, E.P., Slepnev, V.I., David, C., Zhang, X.M., Grabs, D., Sossin, W.S., Bauerfeind, R., Nemoto, Y., and De Camilli. P, A presynaptic inositol-5-phosphatase. Nature 379:353-357, 1996.
160. Salim, K.. Bottomley, M.J., Querfurth, E., Zvelebil, M.J., Gout, I., Scaife, R., Margolis, R.L., Gigg, R., Smith, C.I., Driscoll, P.C., Waterfield, M.D., and Panayotou, G. Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. Embo. J. 15:6241-6250, 1996.
161. Klein, D.E., Lee, A., Frank, D.W., Marks, M.S., and Lemmon, M.A. The pleckstrin homology domains of dynamin isoforms require oligomerization for high affinity phosphoinositide binding. J. Biol. Chem. 273:27725-27733, 1998.
162. Takenawa, T., Itoh, T. Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. Biochim Biophys Acta 1533:190-206, 2001.
163. Lee, C., Kim, S.R., Chung, J.K., Frohman, M.A., Kilimann, M.W., and Rhee, S.G. Inhibition of phospholipase D by amphiphysins. J. Biol. Chem. 275:18751-18758, 2000.
164. Ringstad, N., Nemoto, Y., and De Camilli, P.The SH3p4/Sh3p8/SH3p13 protein family: binding partners for synaptojanin and

dynamin via a Grb2-like Src homology 3 domain. Proc. Natl. Acad. Sci. U.S.A 1997, 94:8569-8574, 1997.
165. Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A.V., Witke, W., Huttner, W.B., and Soling, H.D. Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. Nature 401:133-141, 1999.
166. Rikhy, R., Kumar, V., Mittal, R., and Krishnan, K.S. Endophilin is critically required for synapse formation and function in *Drosophila melanogaster*. J. Neurosci. 22:7478-7484, 2002.
167. Farsad, K., Ringstad, N., Takei, K., Floyd, S.R., Rose, K., and De Camilli, P. Generation of high curvature membranes mediated by direct endophilin bilayer interactions. J. Cell Biol. 155:193-200, 2001.
168. Burger, K.N. Greasing membrane fusion and fission machineries. Traffic 1:605-613, 2000.
169. Ford, M.G., Mills, I.G., Peter, B.J., Vallis, Y., Praefcke, G.J., Evans, P.R., McMahon, H.T. Curvature of clathrin-coated pits driven by epsin. Nature 419:361-366, 2002.
170. Lee, E., Marcucci, M., Daniell, L., Pypaert, M., Weisz, O.A., Ochoa, G.C., Farsad, K., Wenk, M.R., De Camilli, P. Amphiphysin 2 (Bin1) and T-tubule biogenesis in muscle. Science 297:1193-1196, 2002.
171. Richnau, N. and Aspenstrom, P. RICH-a RhoGAP domain-containing protein involved in signalling by Cdc42 and Rac1. J. Biol. Chem. 28:28, 2001.
172. Farsad, K. Direct evidence for a membrane deforming motif in endophilin: implications beyond synaptic vesicle recycling [Ph.D. Thesis]. New Haven: Yale University; 2003.