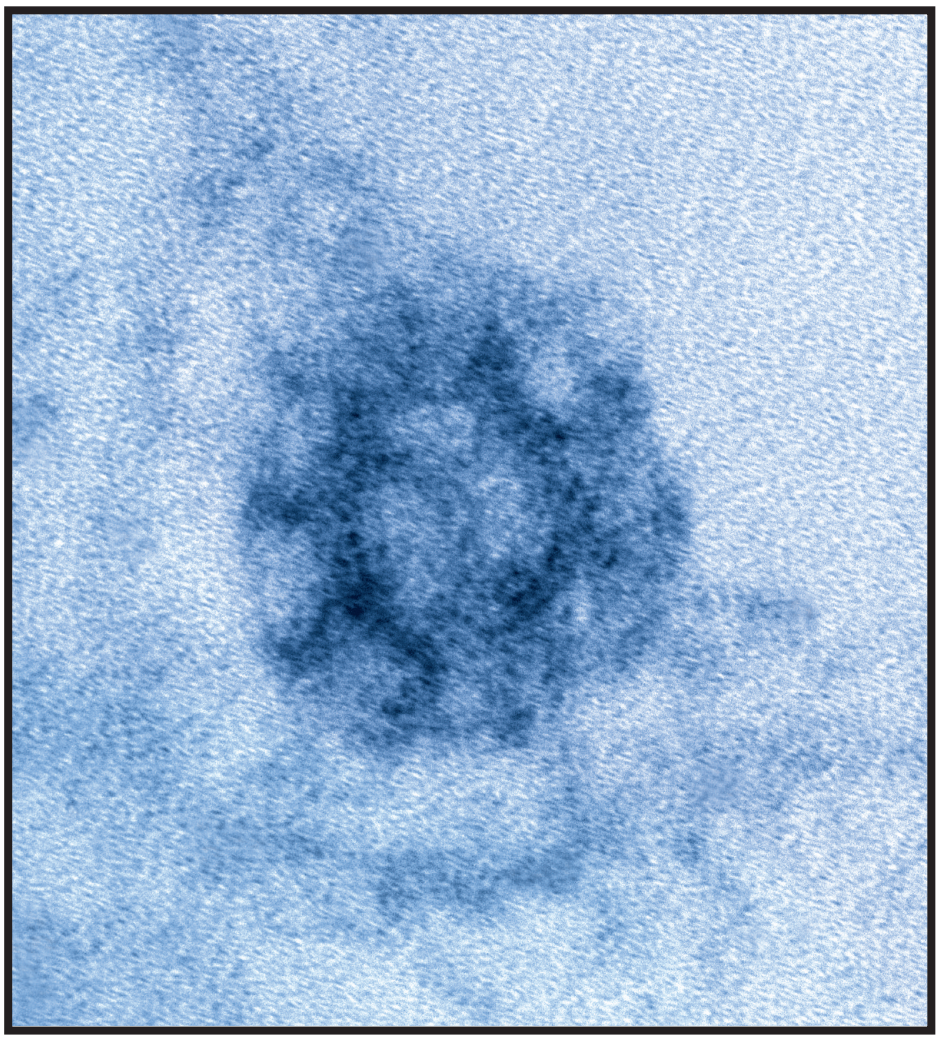
Synaptic vesicle endocytosis studied in a living synapse

Helge Gad



Stockholm **2000**

Nervcellerna i vår hjärna kommunicerar genom att nervimpulser i form av elektriska signaler överförs från cell till cell vid särskilda kontaktorgan i nervcellernas ändar, s.k. synapser. I synapserna finns små vätskefyllda kapslar omgivna av fettmembran, s.k. synapsvesiklar, som innehåller signalsubstans. Nervimpulserna överförs från en nervcell till en annan genom att synapsvesiklarna smälter samman med den ena nervcellens yttermembran. Då frigörs signalsubstansen som kan flyta över till den andra cellen, där det alstras en ny elektrisk signal. Synapsvesiklarna återvinns sedan genom att deras membran tas tillbaka från yttermembranet och bildar nya tomma synapsvesiklar, som fylls på med ny signalsubstans. Denna process är nödvändig för att nervimpulser ska kunna överföras under längre tid.

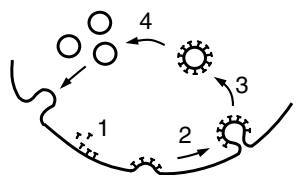
Två alternativa modeller för hur synapsvesikelns membran återvinns har tidigare föreslagits. Enligt den ena smälter synapsvesikelns membran helt samman med yttermembranet. Därefter täcks vesikelmembranet med ett lager av proteinet klatrin, vilket leder till att vesikeln kan återupptas. Enligt den andra modellen smälter synapsvesikeln inte helt samman med yttermembranet. Signalsubstansen släpps i stället ut genom en smal kanal och synapsvesikeln återupptas utan inblandning av klatrin.

Upptaget av membran med hjälp av klatrin-täcken är en process i flera steg. Först binds klatrin till yttermembranet, därefter formas det klatrin-täckta membranet till en vesikel, som sedan knoppas av från yttermembranet. Slutligen avlägsnas klatrin-täcket från den nybildade vesikeln. Troligen måste klatrin-täcket samverka med andra proteiner för att vesikelmembranet ska kunna återupptas.

Målet med denna avhandling är att studera hur synapsvesiklar återvinns och vilken betydelse klatrin-täcket har för detta. Dessutom har proteinerna endofilin, dynamin, amfifysin och synaptojanin undersökts, samt deras roller vid bildningen av klatrin-täckta vesiklar. I undersökningarna användes lättstuderade jättesynapser hos nejonöga. I sådana synapser är det möjligt att spruta in proteiner och med hjälp av elektronmikroskopi se hur de påverkar återupptaget.

Resultaten från experimenten visar att:

- Synapsvesiklar återupptas i huvudsak med hjälp av klatrin-täcken.
- Bildningen av klatrin-täcket på yttermembranet (1) är inte beroende av inflöde av kalciumjoner, vilket tidigare föreslagits. Synapsvesikelns membran är i sig självt tillräckligt för att klatrin-täcket ska bildas.
- Proteinet endofilin är nödvändigt för att öka membranets böjning under klatrin-täcket (2).
- Avknoppningen av den klatrin-täckta vesikeln från yttermembranet (3) är beroende av en samverkan mellan dynamin, amfifysin och endofilin.
- Synaptojanin och dess bindning till endofilin är nödvändig för att slutligen avlägsna klatrin-täcket från den nya vesikeln (4). Möjligen medför endofilins samverkan med dynamin och synaptojanin att processen enbart kan gå framåt.



Sammanfattningsvis har resultaten som presenteras i denna avhandling ökat förståelsen för hur synapsvesiklar tas upp och återvinns. De allmänna mekanismerna bakom vesikel-membrans upptag från cellers yttermembran med hjälp av klatrin-täcken har också klarlagts, vilket ökar förståelsen för hur alla celler i vår kropp kommunicerar med sin omgivning.

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Cover: An IVEM-micrograph of a free clathrin-coated vesicle obtained *in vivo* in the presence of a proline-rich peptide that disrupts the interaction between endophilin and synaptojanin. The peptide (PP19) was microinjected into a lamprey reticulospinal axon followed by action potential stimulation to induce synaptic vesicle recycling.

Magnification: x600000 (the diameter of the vesicle is about 100 nm).

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Abstract

Neurons in the central nervous system communicate by transmitting electrical signals at special sites of contact called synapses. Synapses contain neurotransmitter-filled synaptic vesicles, which fuse with the presynaptic plasma membrane. The neurotransmitter diffuses to the neighboring neuron and triggers a new electrical signal. In order to maintain synaptic transmission, the synaptic vesicle membrane must be retrieved from the presynaptic plasma membrane to be reused for another round of transmitter release. In one proposed model, the synaptic vesicles fuse completely with the plasma membrane and the vesicle membrane is retrieved through clathrin-coated buds. In another model, the synaptic vesicle connects transiently to the plasma membrane and clathrin is not involved in the membrane retrieval.
Clathrin-mediated endocytosis depends on a number of accessory proteins that appear to assist the clathrin-coated intermediate at different steps in the process.

The aim of this thesis was to investigate the role of clathrin-coated buds in synaptic vesicle retrieval. The function of the accessory proteins endophilin, dynamin, amphiphysin and synaptojanin in clathrin-mediated endocytosis was also examined. For this purpose, the giant reticulospinal synapse in lamprey was employed. The organization of this synapse allows acute perturbations of synaptic vesicle recycling.

The following conclusions were drawn from the experiments presented in this thesis:

- Impairment of several clathrin-associated proteins led to an inhibition of synaptic vesicle endocytosis and accumulation of clathrin coated intermediates. This suggests that the main pathway for synaptic vesicle retrieval is through clathrin-mediated endocytosis.
- After temporally dissociating synaptic vesicle release from endocytosis, clathrin-mediated retrieval could be initiated by readding low micromolar concentrations of extracellular Ca^{2+}, without additional action potential stimulation. The results indicate that the synaptic vesicle membrane incorporated in the plasma membrane during exocytosis is a sufficient trigger of synaptic vesicle endocytosis.
- Antibody-mediated disruption of endophilin led to a massive accumulation of shallow clathrin-coated pits on the presynaptic plasma membrane. This suggests that endophilin is required for the invagination of the shallow coated pit, possibly by altering the lipid composition of the coated membrane bud.
- Microinjection of compounds that disrupt the binding of dynamin to amphiphysin and endophilin blocked clathrin-mediated retrieval at the stage of deeply invaginated coated buds with a constricted 'neck'. These data indicate that dynamin and its interaction with amphiphysin and endophilin is essential in the fission of the neck of the coated pit.
- Injection of a peptide blocking the endophilin-synaptojanin interaction and antibodies to the proline-rich domain of synaptojanin induced accumulation of free clathrin-coated vesicles. These results suggest that synaptojanin is recruited to the free coated vesicle by the interaction with endophilin to facilitate the removal of the clathrin coat. Endophilin may be a part of a molecular switch that couples the fission reaction to uncoating and imparts a strict vectorality to synaptic vesicle endocytosis.
- Dynamin was shown to be associated with both early and late stages of coated pits. Antibody-mediated disruption of dynamin blocked synaptic vesicle endocytosis at a stage preceeding clathrin coat formation. These results indicate that dynamin participates at early stages of endocytosis in addition to its role in fission.

The results presented in this thesis have increased the understanding of how synaptic vesicles are retrieved and the molecular mechanisms that govern clathrin-mediated endocytosis, a process important in all animal cells to regulate their response to the external environment.

Keywords: synapse, synaptic vesicles, endocytosis, clathrin, calcium, dynamin, endophilin, amphiphysin, synaptojanin, microinjection, electron microscopy.

This thesis is based on the following original papers that will be referred to in the text by their roman numerals. Abstract and full-text versions of the Paper I-IV can be accessed on the internet by clicking on the blue links.

Paper I: Gad H, Löw P, Zotova E, Brodin L, Shupliakov O. Dissociation between Ca^{2+} triggered synaptic vesicle exocytosis and clathrin-mediated endocytosis at a central synapse. Neuron, 21:607-616, 1998. **Abstract. Full-text.**

Paper II: Shupliakov O, Löw P, Grabs D, Gad H, Chen H, David C, Takei K, De Camilli P, Brodin L. Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions. Science, 276:259-263, 1997. **Abstract. Full-text.**

Paper III: Ringstad N, Gad H, Löw P, Di Paolo G, Brodin L, Shupliakov O, 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. **Abstract. Full-text.**

Paper IV: Gad H, Ringstad N, Löw P, Kjaerulff O, Gustafsson J, Wenk M, Di Paolo G, Nemoto Y, Crum J, Ellisman M, De Camilli P, Shupliakov O, 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. **Abstract. Full-text.**

Paper V: Gad H, Bloom O, Löw P, De Camilli P, Slepnev V, Brodin L, Shupliakov O. A role of dynamin at early stages of clathrin-mediated endocytosis. Manuscript.

Abbreviations:

AP2	adaptor protein 2
ATP	adenosine 5-triphosphate
Ca^{2+}	calcium
CaM	calmodulin
CCD	charge coupled device
CNS	central nervous system
EPSP	excitatory postsynaptic potential
GED	GTPase activator domain
GTP	guanosine 5-triphosphate
GTPγS	guanosine 5-O-(3-thiotriphosphate)
IVEM	intermediate voltage electron microscopy
LPA	lysophosphatidic acid
LPAAT	lysophosphatidic acid acyl transferase
PA	phosphatidic acid
PCR	polymerase chain reaction
PHD	pleckstrin homology domain
PIPs	phosphatidyl inositol polyphosphates
PI(4)P	phosphatidyl inositol 4-phosphate
$PI(4,5)P_2$	phosphatidyl inositol 4,5-bisphosphate
PRD	proline-rich domain
SH3	src homology 3

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Introduction

Neurons in the central nervous system communicate at special junctions called synapses. The chemical synapse is a specialized structure that contains a large number of neurotransmitter-filled 'sacks', the synaptic vesicles. Upon action potential induced depolarization and Ca^{2+} influx, the synaptic vesicles fuse with the plasma membrane at the active zone and release their content into the synaptic cleft (Fig. 1). The neurotransmitter then diffuses across the cleft to the neighboring neuron, binds to receptors on the postsynaptic plasma membrane and triggers an electrical signal. The synaptic vesicle membrane must be retrieved from the presynaptic plasma membrane and recycle back to the active zone in order to preserve synaptic transmission as well as the general organization of the synapse.

Pioneering work on synaptic vesicle endocytosis was done by Heuser and Reese [1]. They used electron microscopy in the frog neuromuscular junction to investigate the involvement of clathrin-mediated endocytosis in synaptic vesicle recycling. After depolarizing the terminals, they were able to label clathrin-coated structures by using a fluid-phase cytosolic marker. They could also follow the appearance of endocytic pits by quick-freeze fixation after a brief depolarization [2]. Heuser proposed that the synaptic vesicles completely collapse into the plasma membrane and the synaptic vesicle membrane diffuse laterally to be retrieved by clathrin-coated invaginations at an area outside the active zone [3]. This model was further supported by other studies demonstrating that clathrin-coated vesicles isolated from brain contain synaptic vesicle proteins and that most components of the clathrin endocytic machinery are enriched in brain. In initial ultrastructural studies, budding was observed from endosome-like cisternae, which led to the conclusion that the recycling synaptic vesicle membrane had to pass through an intermediate endosome compartment before they were ready for release [1]. Recent experiments, however, suggest that the majority of the synaptic vesicles do not pass through the endosome before they are reused for exocytosis [4-6].

Ceccarelli and colleagues proposed an alternative model for synaptic vesicle endocytosis called the 'kiss-and run' hypothesis. They observed omega-shaped profiles at the plasma membrane, which they interpreted as synaptic vesicles connected to the plasma membrane through a small opening [7-9]. In this model, the synaptic vesicles connect transiently to the plasma membrane, release the neurotransmitter through a fusion pore and are then rapidly retrieved without involvement of clathrin. The transient opening of a fusion pore has been observed for large granules in chromaffin cells by capacitance measurements [10,11]. Measurements of the kinetics of synaptic vesicle recycling suggests that there is a slow type of endocytosis with a half-time of 20 seconds and a fast type with a half-time of a few seconds [12-16]. Two distinct routes of synaptic vesicle retrieval have been suggested in the *Drosophila* neuromuscular junction after studying the recovery from the temperature sensitive *shibire*^{ts1} mutation [17]. However, direct evidence for a 'kiss-and-run' type of endocytosis is still lacking and the issue is controversial [3,18-20].

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is one of the major pathways for internalizing membrane proteins in most animal cells. It is for example utilized to regulate the localization and number of receptors on the cell surface. In non-neuronal cells, the clathrin-coated vesicles can have many different sizes, but in the synapse they have a remarkably uniform size. When a specific clathrin binding protein was perturbed, the size of the synaptic vesicles was increased. This correlated with an increase in the quantal size of the neurotransmitter release [6,21,22]. It thus seems that coated vesicle formation in the nerve terminal is a highly regulated process to ensures the formation of uniformly sized synaptic vesicles [20].

Clathrin-mediated endocytosis can be separated into several morphologically distinct steps (Fig. 1). It starts with the binding of the clathrin coat to the plasma membrane, followed by invagination of the clathrin-coated bud, constriction of the neck of the coated pit and fission of the coated bud from the plasma membrane. The components of the coat are then rapidly stripped from the coated vesicle and reused for

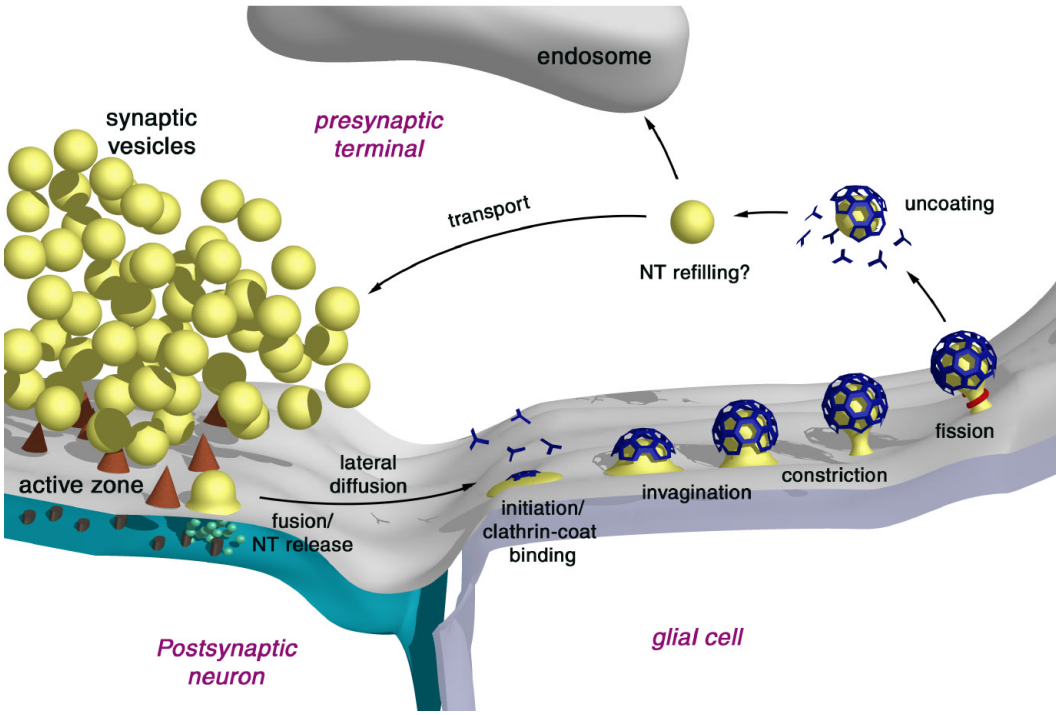


Figure 1. The classical model of synaptic vesicle recycling adapted from [3].
The synaptic vesicles dock at the active zone, collapse into the plasma membrane and release neurotransmitter into the synaptic cleft. The vesicle membrane then diffuses laterally to areas outside the active zone and is retrieved by clathrin-mediated endocytosis at endocytic 'hot-spots'. Clathrin coated vesicle formation involves several morphologically distinct steps, from clathrin coat binding, invagination of the coated bud, constriction and fission of the 'neck' of the bud and the subsequent stripping of the clathrin coat from the free vesicle. The vesicle is then either directly transported back to the cluster of synaptic vesicles or translocated to an intermediate endosomal compartment. The vesicle is refilled with transmitter (NT) at some stage, possible already at the constricted coated pit [141].

another round of vesicle budding.

The core components of the clathrin coat are the three-legged **clathrin triskelion** (Fig. 2A) and the **AP2 adaptor complex** (Fig. 2B). The clathrin triskelia have a strong tendency to self-assemble into a highly ordered polygonal cage of pentagons and hexagons both *in vitro* [23] and under certain conditions, on biological membranes [24,25]. The recognition of the vesicle 'cargo' membrane is most likely mediated by the tetrameric adaptor complex AP2 (Fig. 2B) which binds to transmembrane proteins containing tyrosine-based internalization motifs [26,27].

After the binding of AP2 to the cargo membrane, clathrin may polymerize onto the AP2 template. AP2 also has binding sites for other endocytic proteins, such as dynamin and amphiphysin [28,29]. A critical role of AP2 in synaptic vesicle recycling has been demonstrated in a mutation of the α-subunit of AP2 in *Drosophila melanogaster* [30]. The Ca^{2+} binding protein synaptotagmin 1 binds to AP2 and has been proposed to function as docking site for AP2 [31-34]. The synaptotagmin-AP2 interaction is enhanced by cargo proteins in the plasma membrane and may promote coat recruitment to the plasma membrane [35].

Although clathrin can form cages *in vitro* and clathrin coated intermediates can form simply by adding clathrin/AP2 to purified lipid membranes [24,36], additional factors seem to be necessary for budding from the intact plasma membrane. An array of accessory proteins has been implicated in endocytosis by their direct or indirect association with the clathrin/AP2 coat. Four of the accessory proteins, **dynamin**, **amphiphysin**, **endophilin** and **synaptojanin** (Fig. 2C), are biochemically well-characterized and will be discussed in detail below. They are all enriched in the nerve terminal and bind to each other through SH3-domain interactions which might be regulated by activity-dependent protein dephosphorylations [37-40].

Accessory endocytic proteins

The fission of the neck of the invaginated coated pit requires both ATP and GTP [41] and a protein fission machinery, of which a key component is the GTPase dynamin. Dynamin was first discovered by its ability to form ring-like structures around microtubules [42], although the relevance of this finding is unclear. In the temperature-sensitive *Drosophila* mutant *shibire^{ts1}*, which encodes a mutant form of dynamin defective in GTP-hydrolysis, synaptic vesicle endocytosis was inhibited [43-45]. At the restrictive temperature, nerve terminals showed an accumulation of endocytic pits with electron-dense collars [43,46]. Overexpression in mammalian cells of mutant dynamin defective in GTP binding or hydrolysis, led to an inhibition of clathrin-mediated endocytosis [47-49]. These data indicate that dynamin has a direct role in the budding of clathrin-coated vesicles. Ruptured synaptosomes incubated with GTPγS accumulated coated pits with long dynamin-decorated necks [50]. Purified dynamin self-assembled *in vitro* into rings [51] or spirals around negatively charged liposomes, to which dynamin bound and tubulated [36,52], which implies that dynamin participates in the fission of the neck of coated pits.

Dynamin has several structural domains (Fig. 2C), which include a GTPase domain, a phospholipid binding pleckstrin homology domain (PHD), a GTPase effector domain (GED) and a proline/arginine-rich domain (PRD). The PRD of dynamin has binding sites for the SH3-domain containing proteins amphiphysin [29,53], endophilin [54-56], intersectin/DAP-160 [57,58] and syndapin [59]. The targeting of dynamin to the plasma membrane is thought to be mediated both by SH3-domain interactions [60] and by interactions with phospholipids (see below). Ultrastructural localization has shown that dynamin is associated with both the clathrin coat *per se* and the neck region of coated pits [39,49]. GTP-binding may change the localization of dynamin from the coat to the neck region of the coated pit [61].

Several different models for how dynamin mediates the scission of the coated pits have been proposed [62]. According to the first model, dynamin functions as a mechanochemical enzyme, a 'pinchase', severing the neck of the coated pit upon GTP-hydrolysis [52]. In other experiments, the inter-ring spacing of helical

dynamin increased when GTP, but not GTPγS, was added [63] suggesting that dynamin acts as a 'poppase', expanding the neck of the coated pit until it breaks. Finally, mutations of the dynamin GED, which inhibited the GTPase activity or ring formation, acted stimulating rather than inhibiting on the rate of coated vesicle formation [64]. It was therefore suggested that dynamin when its bound to GTP is a regulator of additional proteins in a fission machinery. None of these models, however, can fully explain the fission process *in vivo*.

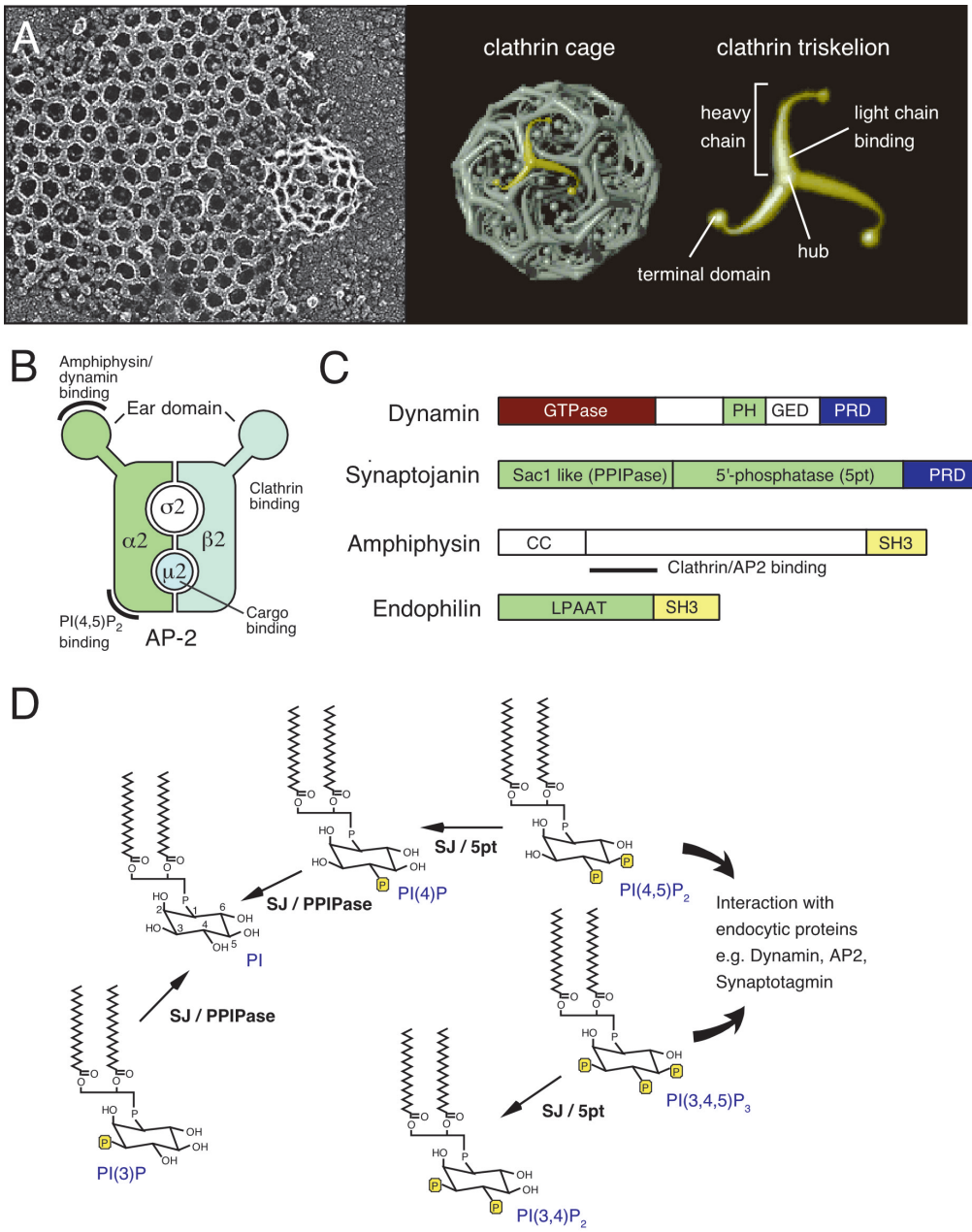
Amphiphysin 1, which forms heterodimers with amphiphysin 2 [65], is also accumulated on clathrin-coated intermediates [39]. The amphiphysin dimer binds to both dynamin and to clathrin/AP2 (Fig. 2C; [40,66,67]). This suggests that amphiphysin may function as an adaptor between dynamin and the clathrin coat [40]. Amphiphysin may also form helical spirals *in vitro* either by itself or together with dynamin [68], indicating that amphiphysin may directly participate in fission.

Lipid-metabolism in clathrin-mediated endocytosis

The lipid composition of the plasma membrane is important in endocytosis, both for protein-lipid interactions and for the biophysical properties of the lipid membrane [69-72]. In perforated cells, preincubation with a PH-domain that selectively binds to **PI(4,5)P_2**, inhibited the AP2 dependent formation of constricted coated pits [73]. Both AP2 and dynamin bind to PI(4,5)P_2 and PI(3,4,5)P_3 *in vitro* [74,75], which is important for targeting both proteins to coated pits [76-79]. Overexpression of mutant dynamin lacking the PI(4,5)P_2 binding PH-domain, inhibited clathrin-mediated transferrin internalization [78-80]. Synaptojanin is a nerve-terminal enriched polyphosphoinositide phosphatase (Fig. 2C) associated with clathrin-coated intermediates [81]. Synaptojanin has a proline rich C-terminus that binds to the SH3-domain containing proteins amphiphysin [82] and endophilin [54-56]. By alternative splicing, a longer from of synaptojanin can be expressed which has additional binding sites to both clathrin, AP2 [83] and eps15, another component of the clathrin coated pits [81,84]. The two enzymatic domains of synaptojanin can dephosphorylate several phosphatidyl inositol polyphosphates (PIPs), including PI(4,5)P_2, PI(3,4,5)P_2, PI(4)P and PI(3)P (Fig. 2D; [82,85]). Brain cytosol from the synaptojanin knockout mice was more potent in generating coated intermediates *in vitro* compared to wild-type mice [86]. In addition, an increased number of coated vesicles were observed in the knockout mice [86]. Morphological analysis of a *C. elegans* mutant of synaptojanin confirmed this finding and also showed defects in other trafficking functions [87]. It was proposed that the dephosphorylation of PI(4,5)P_2 and PI(3,4,5)P_2 by synaptojanin may weaken the binding of AP2 to the lipid membrane and facilitate the uncoating reaction [86].

The SH3-domain containing protein endophilin I is a binding partner for both dynamin and synaptojanin. Endophilin is a **l**yso**p**hosphatidic **a**cid **a**cyl **t**ransferase (LPAAT) required for the formation of synaptic-like vesicles in PC12-cells [88]. Endophilin may convert **lysophosphatidic acid** to **phosphatidic acid**, an activity

Figure 2. Proteins implicated in clathrin-mediated synaptic vesicle formation.
A. Clathrin forms triskelia that can assemble into a highly ordered polygonal coat. Changing the proportion of pentagons and hexagons in the clathrin coat can alter its structure from a flat lattice to a highly curved cage [23,25]. The clathrin triskelion consists of three heavy chains that are linked at the hub and three regulatory light chains. The terminal domain of the heavy chain binds to additional endocytic proteins (see below). **B.** The clathrin adaptor AP2 links the clathrin coat to the cargo proteins in the plasma membrane. Each subunit has binding sites for additional protein as indicated. **C.** Four of the accessory endocytic proteins represented by boxes indicate the approximate size of the different domains. Only one isoform of each protein is represented, although, as a rule, each protein is found in multiple isoforms. Dynamin and synaptojanin contain proline/arginine-rich domains (PRD) that bind to the SH3-domains of amphiphysin and endophilin



[142]. Dynamin has additional domains: a GTPase domain, a pleckstrin-homology domain (PH) and a GTPase effector domain (GED). Domains with lipid metabolizing activity include the 5-phosphatase and Sac1 homology domains of synaptojanin (see below) and the LPAAT activity of endophilin [88]. The coiled-coil domain (CC) of amphiphysin may be responsible for the hetero-dimerization between amphiphysin 1 and 2 [65,143]. Amphiphysin also has binding sites for AP2 and clathrin . **D.** Synaptojanin (SJ) may dephosphorylate several different phosphatidyl inositol polyphosphates [82,85], including $PI(4,5)P_2$ and $PI(3,4,5)P_3$, which interact with several endocytic proteins.

that has been proposed to be important for altering the curvature of the lipid membrane during membrane fission [88]. The CtBP/BARS protein also has LPAAT activity, and seems to be crucial for Golgi membrane fission [89].

Regulation of synaptic vesicle retrieval

Formation of clathrin-coated intermediates follows shortly after Ca^{2+} triggered exocytosis There are conflicting results whether Ca^{2+} might couple these two events. When exocytosis was triggered by α-Latrotoxin, extracellular Ca^{2+} was required to prevent depletion of synaptic vesicles [90,91]. A rapid type of endocytosis in chromaffin cells can be triggered by Ca^{2+} (or Ba^{2+})-influx [92]. Removing the extracellular Ca^{2+} after Ca^{2+}-evoked exocytosis had no effect on the time course of endocyosis [12,93]. Ca^{2+} does not seem to be required for the recovery from the block of endocytosis in *shibire* [94], suggesting that dynamin-mediated fission is not Ca^{2+}-dependent. Studies of synaptosomes have suggested that the Ca^{2+}-calmodulin dependent phosphatase **calcineurin** is required for synaptic vesicle endocytosis, and that elevated intracellular Ca^{2+} levels activate endocytosis [95]. It was proposed that the rise in Ca^{2+} during depolarization may accelerate endocytosis and possibly trigger this event [95]. Calcineurin can dephosphorylate dynamin, amphiphysin and synaptojanin and this may stimulate the GTPase activity of dynamin [96] and promote the formation of endocytic protein complexes [40]. It has been demonstrated both for synaptic vesicles and large granules that Ca^{2+}-influx may increase the rate of endocytosis [13,14]. Other studies have reported an inhibitory effect on synaptic vesicle endocytosis by elevated intracellular Ca^{2+}-levels [97,98]. Moreover, the retrieval of large granules in chromaffin cells was shifted from a classical type of endocytosis to a kiss-and-run mechanism in the presence of high extracellular Ca^{2+}-levels [99].

Aims of the thesis

The main objective of this study is to examine the role of the clathrin-mediated pathway in synaptic vesicle recycling and to investigate molecular mechanisms of this process in a living synapse.The specific aims are to:

1. Investigate the Ca^{2+} dependence of synaptic vesicle recycling and the conditions required for the initiation of clathrin-mediated endocytosis (**Paper I**).

2. Study the effect on synaptic vesicle endocytosis of perturbation of the interaction between the proline-rich domain of dynamin and the SH3-domain of amphiphysin (**Paper II**).

3. Elucidate the role of endophilin in clathrin-mediated endocytosis by acute disruption of its function with antibodies (**Paper III**).

4. Investigate the function of the SH3-domain interactions of endophilin with dynamin and synaptojanin in synaptic vesicle endocytosis (**Paper IV**).

5. Examine the role of dynamin at early stages of synaptic vesicle endocytosis by employing antibodies to disrupt dynamin function (**Paper V**).

Methods

The giant reticulospinal synapse in lamprey-a model system to study synaptic function

One problem in studying synaptic vesicle endocytosis *in vivo* is the relatively small size of most mammalian synapses, which does not permit access to the presynaptic element. In contrast, the lamprey giant reticulospinal synapse is structurally and functionally more suitable and allows acute perturbation of synaptic vesicle recycling. Here follows a short description of the main features of this synapse.

The reticulospinal neurons, located in the lamprey brainstem, have unmyelinated axons that run unbranched from the head to the tail of the animal (Fig. 3).The reticulospinal axons have a diameter of 40 to 80 µm and synaptic contacts (active zones) are made directly on the trunk of the axon. Each release site appears as a single cluster of densely packed synaptic vesicles at a single active zone that often contains gap junctions. Therefore the excitatory postsynaptic potentials (EPSPs) recorded in motor- and interneurons are often composed of both an electrotonic and a chemical component [100]. The active zone and synaptic vesicle cluster may vary greatly in size, from about 0.5 to >2 µm in diameter. Serial sectioning and quantitative analysis has shown a linear correlation between the total number of synaptic vesicles in the cluster and the number of synaptic vesicles in the middle of the synapse [101]. It is thus possible to use the middle section as an index of the entire synapse. Also, the length of the active zone is proportional to the number of synaptic vesicles, which makes it possible to normalize the variations in size between synapses [101].

The size and organization of lamprey reticulospinal axons permits microinjection of antibodies and polypeptides directly into the presynaptic terminal [102]. Since the reticulospinal axon can be regarded as an open cylinder, the same axon can be exposed to different treatments. It is also possible to determine the effect of different concentration of the injected compound by analyzing synapses at different distances from the injection site.

In vitro protein characterizations

Various biochemical experiments, such as molecular cloning, Western blotting, immunoprecipation and affinity chromatography were performed to insure that the protein-protein interactions reported in mammals were conserved in the lamprey nervous system. These experiments were also performed to test whether a polypeptide that inhibited a certain interaction in the mammalian brain was also effective in the lamprey spinal cord.

Molecular cloning

To isolate lamprey orthologues of endophilin and synaptojanin, the amino acid sequences translated from the open-reading frames of human, rat, *Drosophila melanogaster*, and *Caenorhabditis elegans* orthologues were aligned by the clustal method with default parameters using the Clustal X or DNAstar software. The results were compared between the two programs to find regions of high conservation. Degenerate oligonucleotides (20 nt in length) were synthesized based on these regions. A lamprey cDNA library was screened with nested polymerase chain reaction (PCR) using the degenerate oligonucleotides as primers. The obtained DNA product was subsequently used to isolate clones from the same cDNA-library according to standard methods [103]. The 5' and 3' ends of the cDNAs were cloned by the RACE-method using primer pairs derived from the known lamprey sequence and primers either in the library vector or in the polyA-tail. The isolated clones were sequenced at least twice on both strands and the obtained sequences were assembled into a single sequence using the DNAstar software.

Expression of GST-fusion proteins

The regions of interest were amplified by PCR using primers with EcoR 1 sites and a DNA-polymerase with proof-reading activity. The DNA-fragments were purified and subcloned into the pGEX-vector [103]. The vector was transfected into *E.coli* (BL21-cells) and expression was induced with isopropyl-b-D-thiogalactopyranoside for 2-6 h. The cells were then lysed by sonication in the presence of protease inhibitors and insoluble material was removed by centrifugation. The gluthathione-S-transferase (GST)-fusion protein was purified on a glutathione-Sepharose column, dialyzed and concentrated.

Antibodies

Antibodies were generated by immunizing rabbits with either a peptide (anti-endophilin antibody and anti-synaptojanin antibody CAT-1) or with GST-fusion proteins (anti-synaptojanin antibody LSJ-1 and anti-dynamin antibody DG-1). The antigen was coupled to a NHS-activated column that was used to affinity purify the antibodies. They were further purified with protein A Sepharose. The specificity of the antibodies was tested by Western blot using detergent protein extracts from rat brain or lamprey spinal cord and brain. The antibodies were detected using chemoluminesence. Immunoprecipation was performed in De Camilli lab, Yale University, New Haven as described in **Papers III** and **IV**.

Affinity chromatography of tissue extracts

Five grams of rat brain or lamprey spinal cord were minced and homogenized in 10 ml of a buffer containing 20 mM HEPES-KOH pH 7.2, 100 mM KCl, 5 mM EDTA, 4 µg/ml each of leupeptin, pepstatin, antipain and aprotinin, 10 mM benzamidine and 0.4 mM phenylmethylsulfonyl fluoride. A post-nuclear supernatant of the homogenate was prepared by centrifugation at 2,600 rpm for 10 min at 4°C in an SS-34 rotor (Beckman). Triton X-100 was added to the supernatant to a final concentration of 1% and incubated at 4°C for 30 min and then centrifuged for 60 min at 60,000 rpm in a Ti-70 rotor (Beckman), and the resulting high speed supernatant was saved as Triton X-100 extract. Detergent extracts were incubated for 1 hr at 4°C with glutathione-Sepharose loaded with GST fusion proteins. After incubation, the affinity matrix was collected by centrifugation, washed and bound proteins were eluted with SDS-PAGE sample buffer and analyzed by SDS-PAGE [104] and Western Blotting [105]**.** In some experiments detergent extracts were pre-incubated for 1 hr at 4°C with 100 µM PP-19 or PP-15 prior to affinity chromatography.

The lamprey spinal cord preparation

Adult European river lampreys (*Lampetra fluviatilis*) 20-30 cm long were maintained in aerated fresh water tanks at 6-8°C. The animals were anesthetized with MS-222 (tricaine methane sulphonate) and the spinal cord was dissected in physiological Ringer's solution containing 109 mM NaCl, 2.1 mM KCl, 1.8 mM $MgCl_2$, 2.6 mM $CaCl_2$, 4 mM glucose, 2 mM HEPES, 0.073 g/l glutamine, bubbled with 100% O_2 and equilibrated to pH 7.4 at 6°C. Pieces of isolated spinal cord were mounted on Sylgard® with the ventral side up in a glycol-cooled chamber with Ringer's solution maintained at 6-10 °C (Fig. 3).

Manipulations of the extracellular Ca^{2+}

To evoke action potentials in giant reticulospinal axons, the rostral end of the spinal cord was mounted on a bipolar platinum electrode. The efficiency of the stimulation was tested by successively impaling at least four reticulospinal axons in the medial fiber tract near the ventral spinal cord surface with an intracellular microelectrode at the caudal end of the spinal cord. Short (5.5 cm) or long (16 cm) pieces of spinal cord were placed in an experimental chamber of similar length. For the stimulation control, the preparations were fixed (see below) in the experimental chamber after 20 min of stimulated at 20 Hz and stimulation was continued until all action potential activity had ceased. For the zero Ca^{2+} experiment, the preparations were stimulated as above, but the normal Ringer's solution was replaced rapidly by Ca^{2+}-free solution (the $CaCl_2$ was replaced with10 mM EGTA) after 20 min of stimulation and the stimulation was continued as above. Following 90 min in Ca^{2+}-free solution, the preparations were either placed in fixative or placed in Ca^{2+} containing Ringer's solution (2.6 mM $CaCl_2$) before fixation. For measuring the time-course of the appearance of

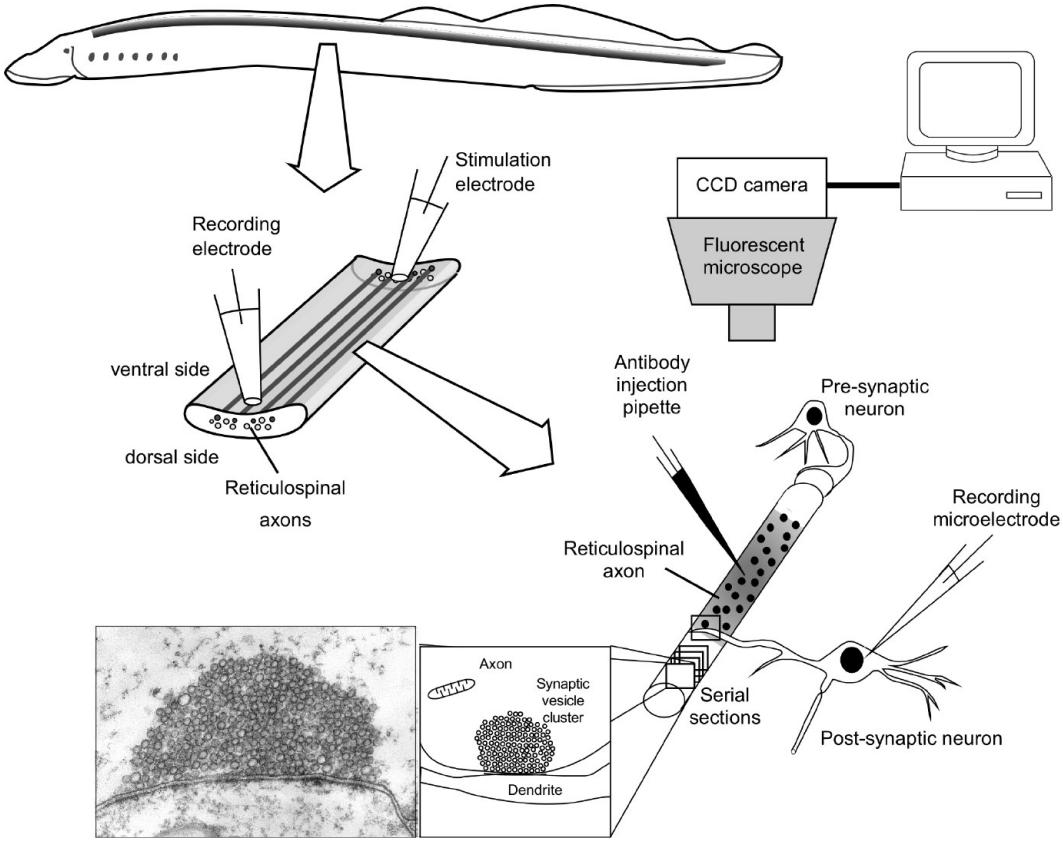


Figure 3. A schematic diagram of the experimental model system.
The spinal cord from adult lampreys was isolated and placed in a recording chamber with the ventral side up. Action potential stimulation was applied with an extracellular electrode and the spikes in reticulospinal axons were recorded either with an extracellular suction electrode or with sharp intracellular electrodes. Fluorescently labeled compounds were microinjected into reticulospinal axons with a glass electrode and the increase in fluorescence was monitored with a CCD-camera connected to a computer. The dark spots in the axon represent labeled synaptic contacts. EPSPs were recorded in postsynaptic spinal neurons. The preparations were fixed and embedded for analysis with electron microscopy. The ultrastructure of synapses at various distances from the injection site was examined in serial ultrathin sections.

coated pits, long pieces (16 cm) of spinal cord were cut into shorter pieces (18-20 mm) when they were still in Ca^{2+}-free solution. The pieces were then placed in normal Ringer's (2.6 mM $CaCl_2$) and incubated for various time periods before fixation. For measuring the minimal Ca^{2+} required to induce coated pits, the spinal cord was cut into smaller pieces as above, incubated for 120 s in Ca^{2+} /EGTA-buffered Ringer's solutions containing varying concentrations of free Ca^{2+}. The actual concentration of free Ca^{2+} was determined with a Ca^{2+}-sensitive electrode according to a previously published method [106]. The stimulation experiments were made at 8°C and the incubations were made at 5°C.

Microinjection experiments

GST-fusion proteins and antibodies diluted in injection buffer (250 mM K acetate and 10 mM HEPES, pH 7.4) were either mixed 10:1 with

Cy5-labeled GST and Cy5-labeled inactive rabbit IgG antibodies respectively or directly labeled with Cy5. Peptides and GTPγS were diluted to a concentration of 30 and 10 mM respectively in injection buffer with 5 μM Texas Red-coupled dextran (molecular weight 3000) added as injection marker. The lamprey spinal cord was isolated and placed in a recording chamber (see above). Reagents were introduced in injection micropipettes (resistance 50-70 MΩ) and injected into the reticulospinal axons (resting membrane potential of–60 mV or lower) with pressure pulses (5-15 psi) of 200 ms duration (Fig. 3). The fluorescence was monitored with a water-cooled charge-coupled-device (CCD) detector mounted on a fluorescence microscope and connected to a personal computer. The effects induced by stimulation were examined in specimens stimulated with electrical pulses applied via an extracellular suction electrode at the caudal end of the spinal cord (Fig. 3). A second electrode, placed at the rostral end, was used to record the spike volleys in the reticulospinal axons. After impaling an axon with the microinjection pipette, test stimuli were applied to verify that the axon was effectively stimulated and to monitor the conducting velocity. The resting membrane potential was monitored during the injection to assure that membrane potential did not increase more than 5 mV. After the injection, the microelectrode was removed and after a period of 5-60 min, stimulation was applied at 0.2 Hz for 60 min (**Paper II**) or at 5 Hz for 30 min (**Papers II-V**). At the end of the stimulation period, the physiological Ringer's solution was replaced with fixative and the preparation was processed for electron microscopic analysis (see below). The effects of injected compounds in resting axons was examined in unstimulated specimens maintained in a low Ca^{2+} Ringer solution (0.1 mM Ca^{2+} and 4 mM Mg^{2+}) for 40-90 min after the microinjection before fixation.

Electrophysiology

The electrophysiological recordings were performed as previously described [102,107]. In short, reticulospinal axons located close to the ventral surface of the spinal cord were impaled with thin-walled microelectrodes containing labeled compounds diluted in 250 mM KAc (see above). Spinal neurons in the lateral cell column were randomly impaled with microelectrodes filled with 3 M KCl to test if they received electrotonic and chemical input. These cells project their medial dendrites to the reticulospinal axons in a nearly perpendicular fashion. Hence the distance between the tips of the stimulating and recording electrodes corresponds to the actual distance between presynaptic injection site and the postsynaptic area. Monitory EPSPs were recorded in current clamp for the spinal neuron before and after microinjection and under different stimulation protocols (**Papers II** and **IV**). The signal was fed through an amplifier and stored into a computer for analysis with Axobasic software (provided by Dr. S. Redman). The total EPSP amplitude was measured between the baseline and the peak of the EPSP. The electrotonic component of the EPSP was measured by applying stimulation at 50 Hz, which eliminates the chemical EPSP. The chemical component of the EPSP was subsequently calculated by subtracting the total APSP from the electrotonic EPSP.

Electron microscopy

The preparations were fixed either in 3% glutaraldehyde plus 0.5% para-formaldehyde in 0.1 M phosphate buffer, pH 7.4 (**Papers I-III**) or in 3% glutaraldehyde plus 4% tannic acid in 0.1 M cacodylate buffer, pH 7.4 (**Papers III-V**) at 5-6°C. In the latter case, 30 min after the fixation the specimens were cut 4-5 mm from the injection site to allow penetration of the fixative. After another 30 min, the specimens were transferred to 3% glutaraldehyde in the same buffer (3-12 hr). The tannic acid fixation was used in the latter part of the papers to better visualize the structure of filaments and proteins. In some cases, the two fixation methods were used in two similar experiments and gave the same results. After the fixation (**Paper I**), the spinal cord was cut in 3-4 mm long pieces at least 7 mm from the cut end to avoid artifacts at the cut ends of reticulospinal axons [108,109]. The specimens were post-fixed in 1% osmium tetraoxide (1 hr), stained *en bloc* in 2% uranyl acetate, dehydrated in ethanol, and embedded in Durcupan ACM resin. Ultrathin serial sections (70-100 nm) from the injection site were cut on an LKB ultratome.

After counterstaining with 2% uranyl acetate and lead citrate, the sections were examined and photographed in a Philips CM12 electron microscope. For the immunogold staining of lamprey reticulospinal axons, see **Paper V**.

Quantitative analysis of injected synapses

The injected axons were always compared to adjacent uninjected axons or to axons injected with control substances. In most specimens, at least 2 different reagents were injected, which had different effects on the ultrastructure of the synaptic regions. The number of synaptic vesicles, the number of coated pits and plasma membrane curvature were quantified in the middle section of the synapses, which can be used as an index of the entire synapse (see above; [101]). The sections were photographed at 13-17.000x and printed on paper with a final magnification of 39-51.000x. The number of synaptic vesicles and coated pits was divided by length of the active zone in μm (measured on scanned micrographs in a Macintosh computer) to normalize for the variation in size between synapses [101]. For quantifying the distribution of different stages coated intermediates, 100-200 coated intermediates were counted on randomly selected serial sections directly in the electron microscope or on printed micrographs. For each free coated vesicle, the lack of connection to other membrane structures were checked on adjacent ultrathin sections. To visualize intact coated vesicles, semithin sections (0.3 and 0.5 μm) were examined using IVEM (**Paper IV**). The membrane curvature of individual randomly selected coated pits (**Paper III**) was calculated by dividing the perimeter length of the membrane of the coated pit with the distance between the edges of the coated pit. The plasma membrane curvature was quantified by measuring the perimeter length between two points on either side of the active zone (AB) and the shortest distance between the two points (CD). The curvature index was calculated by the formula (AB-CD)/(AB+CD) which normalizes the variations in size between synapses [108]. Averages, standard deviations and student's ttests were calculated in the Microsoft Excel and Kaleidograph software.

Miscellaneous procedures

The *in vitro* generation of endocytic coats on synaptic membranes (**Paper III**), the inositol phosphatase activity assay (**Paper IV**), and the liposome experiments (**Paper IV**) were performed in P. De Camilli's lab, Yale University School of Medicine, New Haven, USA.

Results

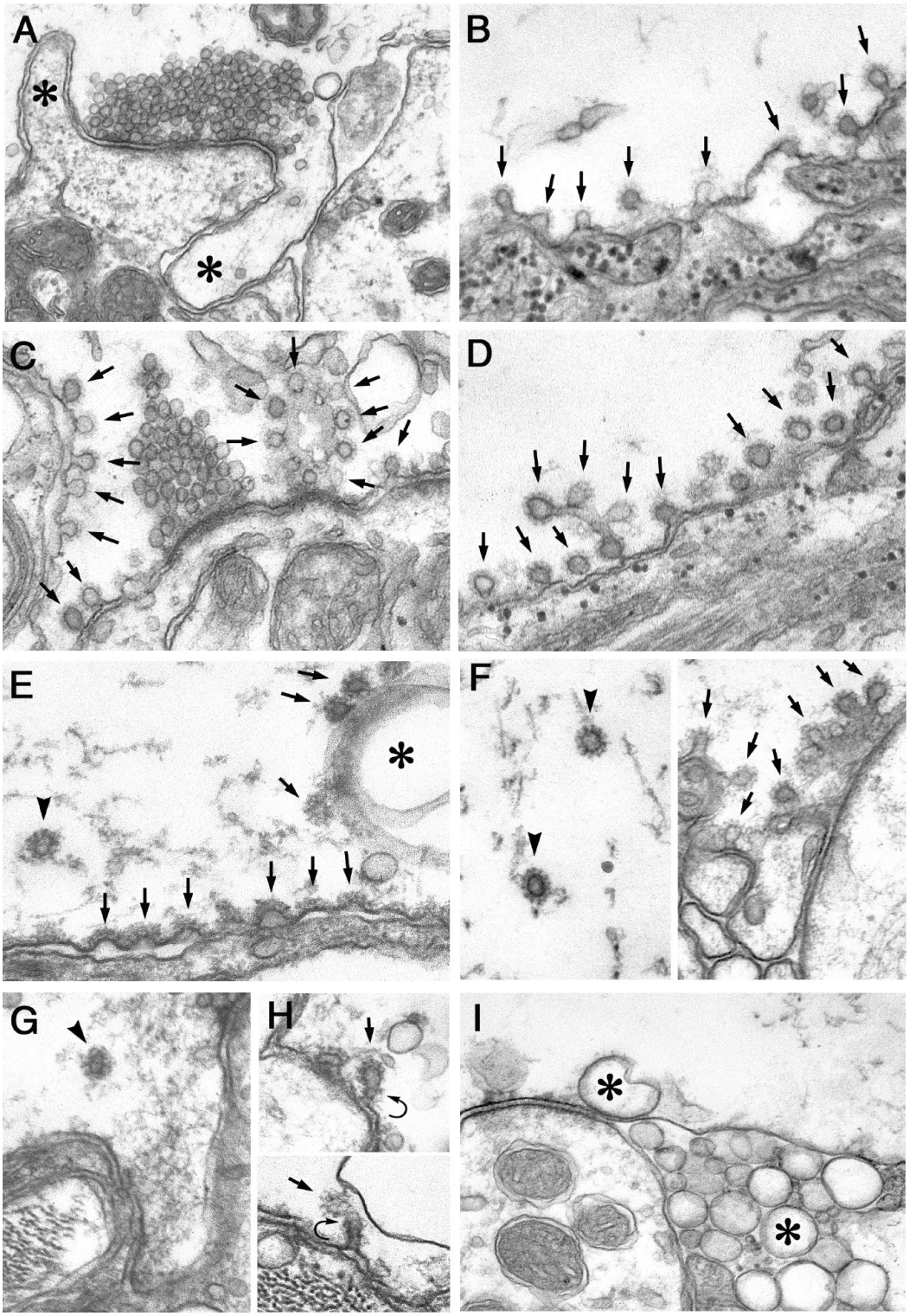
When the reticulospinal axons are stimulated at moderate rates (up to 5 Hz for 30 min) synaptic transmission is sustained and the synaptic vesicle cluster retains its original size [108]. Small expansions of the plasma membrane at the sides of the active zone and a few coated pits are usually present (Table 1). These coated pits have different morphology and can be sub-divided into several classes each representing a distinct stage of coated vesicle formation (**Paper I** and **IV**; cf [2]). This stimulation frequency is probably close to the physiological rate of release to which the reticulospinal synapse is adapted. At higher rates of stimulation (20 Hz for 20 min), however, synaptic transmission is greatly depressed and the synaptic vesicles are depleted [108]. The plasma membrane displays large 'pocket-like' expansions that presumably represent incorporation of synaptic vesicle membrane into the plasma membrane. If the synapse is left at rest for 15 minutes, the synaptic vesicles are restored into a well-organized cluster (**Paper I**; [108]). This tonic stimulation frequency probably represents a condition at which the rate of exocytosis exceeds the rate of endocytosis. Although this tonic stimulation protocol probably exceeds the normal activity rate of this synapse, it can be used as a tool to study synaptic vesicle endocytosis (**Paper I**).

Regulation of synaptic vesicle endocytosis by calcium

The conditions required to initiate endocytosis and its dependence of Ca^{2+} was examined by isolating synaptic vesicle endocytosis from Ca^{2+} evoked exocytosis (**Paper I**). This was archived by applying tonic stimulation (at 20 Hz for 20 min) to lamprey reticulospinal axons. This resulted in a release of a large number of synaptic vesicles (**Paper I**). The physiological Ringer's solution was replaced by Ca^{2+} free solution at the end of the stimulation period and the preparation was maintained at rest for 90 min in Ca^{2+} free solution, more than enough time for a complete recovery from the stimulation. Electron microscopic analysis showed that after this treatment, the number of synaptic vesicles were still reduced. The plasma membrane showed large expansions (Fig. 4A and **Paper I**), presumably due to addition of synaptic vesicle membrane to the plasma membrane. Almost no clathrin-coated buds were observed (Table 1), which indicate that endocytosis was inhibited at a stage prior to the coat formation. Readdition of extracellular Ca^{2+} (2.6 mM), without additional action-potential mediated Ca^{2+} influx, led to a complete recovery of both the synaptic vesicle cluster and the plasma membrane expansions within 15 min (Table 1). To follow the recovery process, extracellular Ca^{2+} was readded for a shorter period before fixation. Numerous clathrin-coated pits of different morphological stages appeared on the plasma membrane around the active zone (Fig. 4B). In the beginning of the recovery phase, early stages dominated while later on, constricted coated pits were more abundant (**Paper I**). Coated pits were always observed either directly on the plasma membrane or on membrane structures connected to the plasma membrane. These results show that synaptic vesicle retrieval through clathrin-mediated endocytosis can be initiated simply by readding Ca^{2+} without additional action potential stimulation.

The minimal extracellular Ca^{2+} level sufficient to activate endocytosis was examined by dissociating exo- and endocytosis and readding Ca^{2+} at different concentrations. Readdition of a Ringer's solution containing 11.2 μM of free Ca^{2+} led to a significant increase in the number of clathrin-coated pits (**Paper I**). Thus, low micromolar concentrations of extracellular Ca^{2+} is sufficient to initiate synaptic vesicle endocytosis.

To address the question whether the extracellular Ca^{2+} added has to pass through voltage-gated Ca^{2+} channels, the channels were blocked by adding cadmium before Ca^{2+} was readded. Cadmium had no effect on the recovery process (data not shown), indicating that voltage-dependent Ca^{2+} channels are not required to activate endocytosis.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Role of accessory endocytic proteins and their interactions at distinct steps of synaptic vesicle endocytosis

Four of the accessory endocytic proteins, dynamin, amphiphysin, endophilin and synaptojanin, have been extensively characterized biochemically in mammalian cells, but their role *in vivo* remains largely unknown. The strategy used in the following papers was to perturb these endocytic proteins and their interactions in the lamprey reticulospinal synapse. Different biochemical experiments were performed to test if the protein-protein interactions were conserved in the lamprey nervous system. Reagents that specifically disrupted these interactions were microinjected into reticulospinal axons and the effects on synaptic vesicle endocytosis were examined using electron microscopy.

Dynamin-amphiphysin interaction

The physiological role of the interaction between dynamin and the SH3 domain of amphiphysin was investigated in **Paper II**. A GST-fusion protein containing the SH3-domain of human amphiphysin (GSTamphSH3) affinity purified one major band from a lamprey spinal cord extract. This band was identified as dynamin by Western blot (**Paper II**) and microsequencing (**Paper V**). However, synaptojanin was not co-affinity purified by GSTamphSH3 from lamprey protein extracts (**Paper IV**). This appeared to be in agreement with the absence of a binding site to amphiphysin in a lamprey synaptojanin orthologue (**Paper IV**).

Fluorescentely labeled GSTamphSH3 was injected into lamprey reticulospinal axons and action potential stimulation was applied (at 0.2 or 5 Hz for 30 min) to induce synaptic vesicle recycling. Ultrastructural analysis showed a massive accumulation of clathrin-coated pits on the plasma membrane surrounding the active zone (Fig. 4C). The vast majority of the coated pits had constricted necks that lacked visible electron-

Figure 4. Perturbations of synaptic vesicle endocytosis in the lamprey reticulospinal synapse.
A. Endocytosis was dissociated from exocytosis by removing extracellular Ca^{2+} at the end of a 20-minute period of high frequency (20 Hz) action potential stimulation (**Paper I**). The preparation was kept in zero Ca^{2+} solution for 90 minutes. Note the expansions of the presynaptic membrane marked by asterisks and the lack of coated pits. **B.** The plasma membrane from a synapse treated as in A and then incubated in normal Ca^{2+}-containing solution for 40 seconds (**Paper I**). Arrows mark clathrin coated pits with varying morphology, representing different stages of coated vesicle formation (see Fig. 1).
C-I. Different reagents were microinjected into reticulospinal axons followed by action potential stimulation at 5 Hz for 30 min to induce synaptic vesicle recycling. Each compound inhibited distinct steps of synaptic vesicle endocytosis. **C, D.** Injection of the SH3-domain of human amphiphysin in C (GSTamphSH3; **Paper II**) and of a 15-mer proline-rich peptide from rat dynamin 1 in D (PP-15; **Paper II**) induced a massive accumulation of uncollared constricted coated pits. **E.** Shallow coated pits were accumulated on the plasma membrane in synapses injected with anti-endophilin antibodies (anti-endo; **Paper III**). Coated intermediates of later stages were seen on deep invaginations of the plasma membrane (asterisk) and occasionally also coated vesicles (arrowhead). **F.** In synapses injected with a peptide from the proline-rich domain of synaptojanin (PP-19; **Paper IV**), a large number of free coated vesicles were observed along with uncollared, constricted coated pits. **G.** Free coated vesicles were also seen after injection of anti-synaptojanin antibodies (LSJ-1; **Paper IV**). **H.** Accumulation of coated pits with longer 'necks' surrounded by electron-dense collars (curved arrows) in synapses injected with the SH3-domain of endophilin (GSTendoSH3; **Paper IV**). **I.** Injection of anti-dynamin antibodies (DG-1; **Paper V**) blocked synaptic vesicle recycling before coat formation. Asterisks indicate synaptic vesicle membrane internalized into the plasma membrane. GST and non-immune antibodies were also injected and had little or no effects as compared to uninjected synapses (see also Table 1).

dense rings. The synaptic vesicles were greatly reduced in number and the plasma membrane had expanded as compared to uninjected synapses in the same preparation (Table 1). At 5 Hz stimulation, the active zone was fragmented and membrane invaginations bearing coated pits were seen.

GSTamphSH3 was also injected in reticulospinal axons maintained at rest (in low Ca^{2+} solution). Synapses in these axons were ultrastructurally very similar to resting uninjected synapses.

Several controls were used. Reticulospinal axons were injected with a mutant form of GST-amphSH3 lacking binding to lamprey dynamin (**Paper II**), and stimulation at 5 Hz for 30 min was applied. No depletion of the synaptic vesicle cluster or any plasma membrane expansion was observed, although a modest increase in the number of coated pits were observed. The number of coated pits was, however, 8 times smaller as compared to the wildtype form of GSTamphSH3 and both early and late stages of coated pits were observed (Table 1). This suggests that the mutation in GSTamphSH3 greatly reduced its binding to dynamin, but that it still had a small effect on endocytosis. Additionally, GST alone or the SH3 domain of the postsynaptic protein SAP90 was injected in lamprey axons subjected to the same stimulation protocol as above. No effects on the ultrastructure were seen neither with GST nor SAP90 compared to uninjected synapses (Table 1 and **Paper II**).

A peptide from the amphiphysin binding site of rat dynamin (a 15-mer proline-rich peptide, PP15; [53,110] was also used to perturb the dynamin-amphiphysin interaction. PP15 inhibited the affinity purification of lamprey dynamin by GST-amphSH3 (**Paper II**), but had no effect on the binding between dynamin and endophilin in the lamprey (**Paper IV**). Microinjection of PP15 into lamprey nerve terminals subjected to stimulation had qualitatively similar effects as GSTamphSH3. A stimulus dependent accumulation of deeply invaginated coated pits with constricted necks (of stage 3) and a reduction of the synaptic vesicles were observed (Table 1).

The effect of GSTamphSH3 on neurotransmitter release was investigated by measuring the excitatory postsynaptic potential (EPSP) in lamprey spinal neurons. At low rate of release, there was no effect on the chemical component of the EPSP amplitude. However, with high frequency stimulation, an enhanced depression was observed compared to the amphSH3 mutant, consistent with an inhibition of endocytosis by GSTamphSH3. Thus the results from **Paper II** show that disruption of the dynamin-amphiphysin interaction inhibits synaptic vesicle endocytosis at a stage after constriction but proceeding fission of the invaginated coated pit.

Endophilin and its binding to dynamin and synaptojanin

To study the function of endophilin I (SH3p4) in synaptic vesicle endocytosis, antibodies against the SH3-domain of rat endophilin were tested on lamprey spinal cord tissue (**Paper III**). The anti-endophilin antibodies recognized a single band of appropriate size on Western blot and co-immunoprecipitated endophilin, dynamin and synaptojanin. A lamprey orthologue of endophilin I was isolated from a lamprey cDNA-library. The lamprey sequence was highly similar to mammalian endophilin, indicating a high degree of conservation of endophilin in lamprey.

In axons microinjected with anti-endophilin antibodies and maintained at rest, no evident ultrastructural changes were observed (**Paper III**). Stimulation at 5 Hz for 30 min dramatically changed the structure of the nerve terminal in antibody-injected axons. The synaptic vesicle pool was reduced as compared to adjacent uninjected axons and the plasmalemma exhibited expansions and endosome-like infoldings, which indicate that endocytosis was inhibited. A large number of clathrin-coated pits accumulated on the plasma membrane adjacent to the active zone (Table 1). In contrast to the deeply invaginated coated pits in GSTamphSH3 injected axons (**Paper II**), the coated pits in endophilin antibody injected axons were all of an early type, i.e. with a shallow curvature (Fig. 4D). As in uninjected control axons, synapses in axons injected with non-immune 'control' rabbit IgG antibodies (**Papers IV** and **V**) had a large synaptic vesicle cluster and a limited number of coated pits of different morphology (Table 1).

The effects of different concentrations of the anti-endophilin antibodies were examined by analyzing coated pits in synapses at various distances from the injection site. The membrane curvature of individual coated pits increased with decreasing antibody concentration, i.e. they became more invaginated. When the concentration decreased further, the variability in membrane curvature between individual coated pits increased. Thus disruption of endophilin function inhibited synaptic vesicle endocytosis and prevented the coated pits from obtaining curvature.

In some of the synapses injected with endophilin antibodies, 'endosome-like' infoldings of the plasma membrane was also observed (see also **Paper I**). The infoldings exhibited deeply invaginated coated pits on their surface and occasional coated vesicles were also noted (Fig. 4E; **Paper III** and **IV**). Synapses lacking' endosome-like' infoldings only contained coated pits with a shallow curvature.

In synaptic membranes incubated with brain cytosol, ATP and GTPγS [4], depletion of endophilin from the cytosol inhibited the formation dynamin-coated tubules, but not the formation of clathrin coats (**Paper III**). Furthermore, the synaptic membranes were stained with anti-endophilin antibodies and analyzed with immunogold electron microscopy. Endophilin immunoreactivity was primarily located to dynamin coated tubules, which presumably corre-

Experimental condition	Synaptic vesicles	Coated pits	PM curvature index	Coated intermediates per stage (% of total)					Primary stage of inhibition	Paper
				stage 1	stage 2	stage 3	stage 4	stage 5		
Resting	322±76	0.1±0.3	0.05±0.02							I to V
20 Hz stim.	80±43	7.9±4.6	0.33±0.10							I
20 Hz stim. +zero Ca^{2+}	120±27	0	0.30±0.08						initiation	I
Readdition of Ca^{2+} /40 sec	123±21	8.7±4.0	ND	26	28	42	4	0		I
Readdition of Ca^{2+} /15 min	233±53	0.8±1.1	0.04±0.01							I
Injections, 5 Hz stim.										
GSTamphSH3	58±32	45.8±6.0	0.35±0.07	0	0	100	0	0	fission	II
PP15	156±30	29.1±3.3	0.09±0.17	5	5	90	0	0	fission	II
anti-endophilin	33±24	15.8±6.8	*	100	0	0	0	0	invagination	III
PP19	45±35	15.9±4.3†	0.46±0.10	6	5	39	1	50	fission and uncoating	IV
anti-synaptojanin	139±25	1.7±1.6†	0.26±0.08	10	12	48	5	26	uncoating	IV
GSTendoSH3	47±20	12.8±6.4	*	2	1	14	82	0	fission	IV
anti-dynamin	7±3	3.5±2.9	0.20±0.14	54	19	25	2	0	initiation	V
Controls, 5 Hz stim.										
uninjected	270±58	0.8±1.0	0.06±0.04	17	17	64	3	0		IV
GSTamphSH3mut	174±49	5.8±1.3	0.05±0.05	19	16	65	0	0		II
GST	234±26	1.1±0.5	0.02±0.01	15	13	73	0	0		II, IV
Nonimmune IgG	251±36	1.5±0.8	0.04±0.02	14	12	69	5	0		IV, V

Table 1. Summary of the quantitative data different perturbation experiments.
The upper part of the table shows experiments when tonic stimulation at 20 Hz for 20 minutes were applied to exhaust the recycling machinery. The middle and lower parts show experiments with a moderate stimulation frequency (5 Hz for 30 min), a rate at which the vesicle pool is maintained by recycling [108]. The number of synaptic vesicles and coated pits as well as the expansions of the plasma membrane (PM curvature index) was quantified in the middle of the synapse and normalized to the size of the active zone. The coated intermediates were classified into five different stages according to their morphology, shown in the table as schematic drawings. For the number of samples for each experiment refer to the original papers.

*) Due to large 'endosome-like' invaginations of the plasma membrane, the PM curvature index was difficult to quantify.
†) Free coated vesicles are not included (for details see **Paper IV**).

sponds to the neck region of the coated pit. Surprisingly, gold particles were virtually absent on clathrin-coated structures (**Paper III**).

Taken together, these findings indicate that endophilin has functions at later stages of clathrin-mediated endocytosis in addition to its role in the invagination process. This hypothesis was further investigated by studying the role of the interaction of the SH3-domain of endophilin with dynamin and synaptojanin (**Paper IV**). First, a lamprey synaptojanin orthologue was isolated by PCR amplification and screening of a lamprey CNS cDNA library. The inositol polyphosphatase domains were well conserved, and the essential residues in the active sites were identical to those in mammalian synaptojanin. Purified lamprey synaptojanin dephosphorylated PI(4,5)P_2 and PI(3,4,5)P_3 confirming the similarity in catalytic activity between lamprey and mammalian synaptojanin. The proline-rich domain had less similarity although one of the endophilin binding sites [110] was conserved. A 19-mer proline rich peptide, named PP19, was synthesized based on this site. Next, PP19 was bound to a matrix and incubated with a lamprey spinal cord protein extract. One major band was affinity purified that was identified as endophilin I by Western blot (**Paper III**). In other experiments, the affinity purification of lamprey dynamin and synaptojanin by full-length rat endophilin I or by the SH3-domain of rat endophilin I was inhibited by PP19 (**Paper IV**).

Microinjection of PP19 into lamprey reticulospinal axons and action potential stimulation (at 5Hz for 30 min) dramatically changed the ultrastructure of the nerve terminal. Similar to GSTamphSH3, the presynaptic plasma membrane had 'pocket-like' expansions with tubular infoldings and the synaptic vesicle pool was almost totally depleted (Table 1). In contrast to GSTamphSH3 and PP15, numerous free clathrin-coated vesicles were present in PP19 injected axons. They were observed close to the active zone and also at greater distances from the synaptic area, even in the center of the giant axon (Fig. 4E). The coated vesicles were traced in serial ultrathin sections as well as in thicker sections containing the entire vesicle, which showed that they were indeed isolated from other membrane structures. Normally, free coated vesicles are not present in unperturbed stimulated reticulospinal synapses, most probably due to a rapid uncoating of the vesicles immediately after fission [111]. Free coated vesicles were not found in stimulated synapses injected with GSTamphSH3, PP15, GST, non-immune IgG antibodies or anti-dynamin antibodies (Table 1 and **Paper V**). A great number of coated pits accumulated on the plasma membrane in PP19-injected synapses and the majority of these were deeply invaginated (Fig. 4E, Table 1 and see below). An actin-like filamentous matrix was also present laterally to the active zone.

The occurrence of free coated vesicles could be explained by an inhibition of uncoating, although other explanations, such as 'reversed coating' of synaptic vesicles or enhanced vesicle turn-over, are feasible. However, PP19-injected synapses maintained at rest (in low Ca^{2+} Ringer) displayed a normal appearance identical to that of uninjected synapses. This suggests that a reversed coating of synaptic vesicles is unlikely. At low frequency stimulation of reticulospinal axons, the EPSP amplitude in spinal neurons remained unchanged after PP19 injection (**Paper IV**). The recovery of the EPSP amplitude after intense (50 Hz) stimulation was, however, impaired. This speaks against a possible enhanced synaptic vesicle turnover and favors the former hypothesis stated above.

It seems plausible that the accumulation of coated vesicles by PP19 could be due to impaired recruitment of synaptojanin to the membrane. This would prevent dephosphorylation of PIPs and retard the stripping of the coat. To examine this further, rat brain cytosol was added to protein-free liposomes and the bound proteins were detected by Western blot. Incubation with PP19 substantially reduced the amount of bound synaptojanin, while the binding of other proteins showed little reduction (**Paper IV**). To obtain further support for the role of endophilin-synaptojanin interaction in the uncoating reaction, antibodies against the proline-rich domain of lamprey synaptojanin (LSJ-1) were used. The antibodies recognized two bands of 145 and 170 kDa on Western blot of lamprey nervous tissue, similar in size to the reported isoforms of mammalian synaptojanin 1 [82]. Synapses in stimulated axons microinjected with LSJ-1 exhibited a number of morphological features: a depletion of

synaptic vesicles, an expansion of the presynaptic membrane (Table 1), an induction of cytoskeletal filaments and the presence of free coated vesicles (Fig. 4G). As with PP19, the coated vesicles were traced on serial sections, confirming that they were not connected to other membrane compartments (**Paper IV**). Although they were not so numerous as in PP19 injected synapses, coated vesicles were found in every synapse examined. Thus, it seems likely that the recruitment of synaptojanin by endophilin is needed for proper uncoating of coated vesicles.

The combined accumulation of coated vesicles and invaginated coated pits in PP19 injected axons, suggest that PP19 did not only perturb uncoating but also affected the fission reaction. This was examined further using the SH3-domain of endophilin (GSTendoSH3) which *in vitro* bound to dynamin and synaptojanin in lamprey spinal cord extracts. As in the case of PP19, microinjection of GSTendoSH3 led to a reduction in the number of synaptic vesicles (Table 1) and expansion of the plasma membrane. Also, clathrin-coated pits, which were mostly deeply invaginated, accumulated at the plasma membrane. Two major differences from the effects of PP19 were, however, observed. Free coated vesicles were not detected in axons injected with GSTendoSH3 and the majority of the coated pits had an elongated neck that was often surrounded by one or more electron-dense ring-like structures (Fig. 4H). This was reminiscent, but not identical, to the electron-dense rings induced by GTPγS both in rat synaptosomes [50] and in lamprey reticulospinal axons (**Paper IV**). Taken together, the effects of PP-19, GSTendoSH3 and GTPγS on clathrin-coated pits provide morphological support for a role of endophilin and its SH3 domain also at the fission step.

Additional functions of dynamin

Dynamin is localized to both the clathrin coat as well as to the neck region of coated pits (see Introduction), which implies that dynamin may have several functions in endocytosis. Immunocytochemistry was used to test whether dynamin is associated with early stages of coated pits (**Paper V**). Antibodies (DG-1) that specifically recognized dynamin in the lamprey CNS were used to stain reticulospinal axons subjected to tonic stimulation (30 mM K^+ on ice; [108]). The dynamin immunogold labeling was associated with both shallow coated pits ('early' type) and deeply invaginated coated pits ('late' type).

The function of dynamin was disrupted by injection of anti-dynamin antibodies into reticulospinal axons. The antibodies did not alter the synaptic morphology in axons maintained at rest (**Paper V**). In contrast, stimulation of the axons at 5 Hz induced a massive depletion of the synaptic vesicles and expansion of the plasma membrane (Fig. 4I), similar to the effects of e. g. GSTamphSH3 and PP19 (Table 1). However, they differed markedly with regard to the occurrence of coated pits. Whereas injection of GSTamphSH3, endophilin antibodies and PP19 induced a massive increase in the number of coated pits, only a small number were present after injection of anti-dynamin antibodies (Table 1). Also, the coated pits in GSTamphSH3 and PP19 injected axons were mostly deeply invaginated, whereas shallow coated pits predominated in dynamin antibody-injected axons. Free clathrin-coated vesicles were not detected in dynamin antibody-injected axons. The data presented in **Paper V** thus suggest that dynamin also participates at early stages of clathrin-coat formation in addition to the fission reaction.

Discussion

The data presented in this thesis consistently show that the main pathway for synaptic vesicle retrieval is through clathrin-mediated endocytosis. A stimulus dependent reduction of the number of synaptic vesicles was observed after injection of reagents that perturbed specific components of the clathrin endocytic machinery. These include GSTamphSH3 and PP15 (**Paper II**), dynamin antibodies (**Paper V**), endophilin antibodies (**Paper III**), PP19, GSTendoSH3 and synaptojanin antibodies (**Paper IV**), which impaired the function of dynamin, amphiphysin, endophilin and synaptojanin (Table 1). The decrease in the number of synaptic vesicles was in most cases accompanied by expansion of the presynaptic plasma membrane. This probably represent synaptic vesicle membrane that has been incorporated and trapped into the plasma membrane [1,108].

In most microinjection experiments, a small proportion of synaptic vesicles still remained, whereas in dynamin antibody injected synapses, nearly all of the vesicles were depleted. This could be interpreted as if these reagents did not completely inhibit the function of their target protein, and endocytosis could still proceed although at much slower rate. Complete removal of a core component of the clathrin coat, AP2, led to a total depletion of the synaptic vesicles together with plasma membrane expansions [30], which supports this interpretation. Alternatively, there might be other retrieval mechanisms that are dynamin but not clathrin dependent. A dynamin dependence but clathrin independence was observed for rapid endocytosis of large granules in adrenal chromaffin cells [92]. However, the physiological role of the excess retrieval by rapid endocytosis is unclear and might not be used under normal conditions of membrane cycling [14,112].

For two of the injected reagents, GSTamphSH3 and PP19, the effect on neurotransmitter release was analyzed by recording the response in the postsynaptic neuron (**Papers II** and **IV**). In neither case was there any effect on synaptic vesicle release at low stimulation rates (0.2 Hz). At higher stimulation rates, however, there was an enhanced depression of the neurotransmitter release. This is in agreement with many other studies that have shown that synaptic vesicle endocytosis is essential for sustained synaptic transmission. The onset of the depression when switching from low to high stimulation rates was faster than would have been expected from a defect only in endocytosis (**Paper II**). Similar effects were observed in the *shibire^{ts1}* mutant, where an inhibition of the postsynaptic respons occurred within 10-20 msec at the restrictive temperature [113]. In the GSTamphSH3-injection experiment, a division and expansion of the active zone area can explain this rapid depression. This would perhaps lower the density of the Ca^{2+}-channels and change the release probability.

Clathrin-mediated endocytosis or 'kiss-and-run'?

The massive accumulation of clathrin-coated intermediates after disruption of the SH3-domain interactions of amphiphysin (**Paper II**) and endophilin (**Paper IV**) provides evidence that clathrin-coated vesicles are *bona fide* intermediates in synaptic vesicles recycling. This is also supported by the induction of coated intermediates when endocytosis was re-started after the blockade in zero Ca^{2+} (**Paper I**). When Ca^{2+} was readded, there seemed to be no increase in endosome-like structures, which makes it unlikely that a 'bulk' uptake of membrane is involved. Omega-shaped uncoated membrane pits have been observed in the neuromuscular junction, both in quick-freeze fixed synapses [8] and in the dynamin *shibire^{ts1}* mutation [9,43]. In the latter case, the necks of the endocytic pits often contained electron dense collars. Uncoated endocytic pits were not observed in the injected or uninjected reticulospinal synapses that were examined. However, this does not rule out their presence, since it may be hard to visualize them in chemically fixed synapses.

One intriguing observation made in various synapses is that during increasing rates of synaptic vesicle release, there was no corresponding increase in the number of coated pits. This has been interpreted either as an increased rate of endocytosis at higher rate of release and a short life time of the coated intermediates [3], or as an argument for alternative routes of endocytosis [114]. The increase in the number of coated pits after inhibition of clathrin-coated vesicle formation (Table 1) shows that it is possible to reveal the otherwise short-lived coated intermediates. Although the number of coated pits did not increase further with increasing rates of stimulation after GSTamphSH3 injection (Paper II), more synaptic vesicle membrane was trapped in the plasma membrane at the higher rate of stimulation. These results also suggest that there is a limitation in the maximum number of coated intermediates able to form. When a large amount of coated pits are formed the pool of available coat proteins could be depleted and limit further coat formation. This assumption is consistent with measurements of the residence time in the plasma membrane of the synaptic vesicle protein VAMP [115]. It was concluded from that study that endocytosis appears to be a saturable process.

The data presented in this thesis thus implies that synaptic vesicles are mainly retrieved through coated intermediates. It is difficult, however, to rule out that alternative pathways, such as 'kiss-and-run' type of endocytosis, may occur in parallel under certain conditions. In the classical model of synaptic vesicle recycling, the newly formed coated vesicle was assumed to fuse with an endosome compartment where an additional clathrin mediated budding event would occur [2]. The involvement of large cisternae in the recycling after reversible block of endocytosis in *shibire*^{ts1} flies have been reported [17,46] but it is unclear whether these represent reversible infoldings of the plasmalemma or true endosomes. Serial section analysis of lamprey reticulospinal synapses injected with several different reagents (Fig. 4) showed that the coated pits seen were solely present on the plasma membrane and on membrane cisternae with a narrow connection to the plasma membrane. Analysis of serially sectioned 'endosome-like' structures in lysed nerve terminals have also shown that they are connected to the plasma membrane [4]. Moreover, studies of the kinetics of synaptic vesicle endocytosis favors recycling without an intermediate compartment [5]. Further evidence for a direct recycling pathway was obtained after perturbation of the clathrin adaptor AP180, which increased the size of the synaptic vesicles [6,21,22]. This suggests that the endosome-like intermediates of nerve terminals originate from infoldings of the plasma membrane and that clathrin-mediated budding may take place in parallel from infolded and intact regions of the plasmalemma.

Why does budding from the plasma membrane require so many accessory proteins?

The retrieval of proteins in the plasma membrane by clathrin coats, either membrane receptors in non-neuronal cells or synaptic vesicle proteins, requires a large array of endocytic proteins [116]. In contrast, COP II coated vesicle mediated budding from the ER appears to require less accessory proteins [117]. Whether this difference in complexity is real or not, several explanations are plausible.

Flat clathrin lattices and shallow coated pits are observed predominantly when clathrin coat components are added to the plasma membranes attached to a surface, while clathrin coats formed *in vitro* or on isolated membranes tends to form coated pits with a higher curvature [3,24]. Interestingly, when disrupting the function of endophilin (**Paper III**), coated pits with a shallow curvature were observed at the intact plasma membrane while other parts of the plasma membrane that were deeply invaginated exhibited highly curved coated intermediates. This may mean that it is more difficult to induce curvature of the intact plasma membrane as compared to an intracellular compartment. The lipid composition is different in the plasma membrane as compared to intracellular compartments. Membrane microdomains with a different lipid composi-

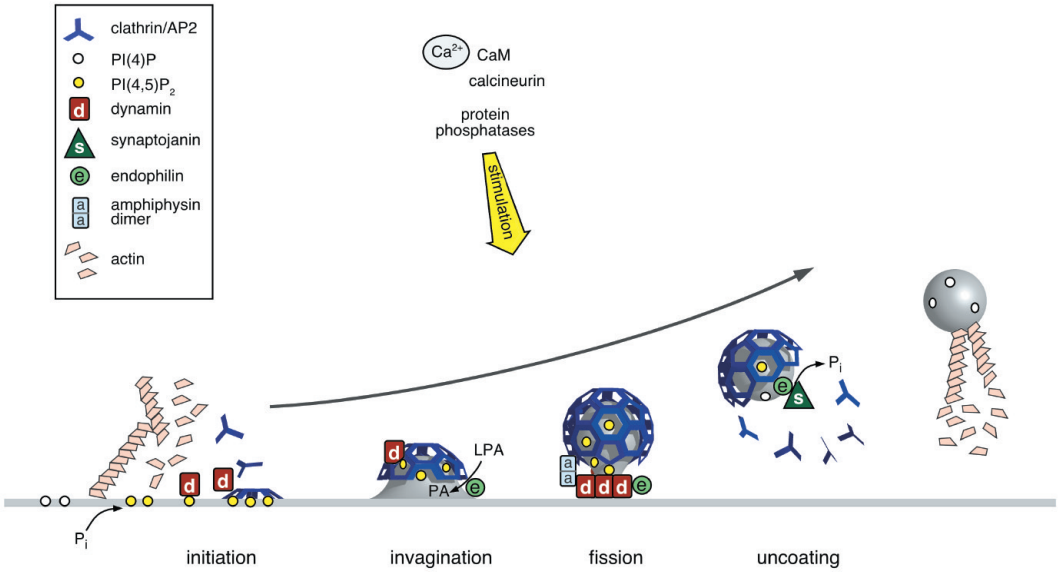


Figure 5. A hypothetical model describing molecular mechanisms in clathrin-mediated synaptic vesicle endocytosis.
Initiation: Phosphorylation of PI(4)P to PI(4,5)P_2 promotes binding of both dynamin and AP2 to the synaptic vesicle membrane incorporated into the plasma membrane. Dynamin can interact with effector proteins (e. g. profilin) that may destabilize the cortical actin cytoskeleton, that would allow clathrin coat polymerization. The clathrin/AP2 coat also binds to synaptic vesicle-associated transmembrane proteins [27] and to synaptotagmin [31]. **Invagination:** Endophilin may promote the invagination of the shallow clathrin coat bud by recruiting lysophosphatidic acid (LPA) from the cytoplasm to the membrane. LPA is then converted to phosphatidic acid (PA) and incorporated into the outer leaflet of the lipid bilayer. This would lead to an assymetry between the inner and outer leaflet and consequently increase the membrane curvature of the coated bud. **Fission:** The amphiphysin dimer binds to both clathrin/AP2 and dynamin. Dynamin and amphiphysin may oligomerize into a collar around the neck of the constricted coated bud. Endophilin also binds to dynamin and the enzymatic activities of the two proteins may mediate the scission of the endocytic bud from the plasma membrane. **Uncoating:** Endophilin releases dynamin after the fission and recruits synaptojanin to the free coated vesicle. Synaptojanin dephosphorylates PI(4,5)P_2 into PI(4)P which weakens the interaction of AP2 to the vesicle membrane. The clathrin/AP2 coat is then stripped from the vesicle by the Hsc70/auxilin complex [139,140].
Filamentous actin could be involved in the transport of the synaptic vesicle back to the release site. Several of the endocytic proteins can be dephosphorylated by several protein phosphatases, such as the Ca^{2+} and calmodulin (CaM)-dependent phosphatase calcineurin. This could promote the assembly of protein complexes that would perhaps increase the rate of endocytosis.

tion compared to the surrounding plasma membrane have been implicated in many trafficking events [71,118]. The synaptic vesicle membrane protein synaptophysin is associated with cholesterol enriched microdomains, so called detergent-resistant lipid rafts [119]. Cholesterol depletion halts clathrin-mediated endocytosis at the stage of shallow coated pits [120,121], the same stage that was impaired after endophilin antibody injection (**Paper III**). A protein machinery might be required to change the membrane of coated pits at the plasma membrane to assist in the membrane bending and to facilitate the fission reaction (Fig. 5). Endophilin I could have such functions as indicated by the block of the invagination after endophilin antibody injection

(**Paper III**), inhibition of the fission reaction after SH3 domain impairment (**Paper IV**) together with the LPAAT activity of endophilin [88]. Although little is known about the exact mechanism how this is accomplished, there are several possible scenarios. The conversion of lysophosphatidic acid (LPA) to phosphatidic acid (PA) has been suggested to cause an asymmetry between the lipid bilayers and regulate membrane bending. LPA is a highly water soluble lipid and if LPA would be recruited from the cytosol and converted to PA, the cytosolic membrane leaflet area would increase. This would lead to a more positive (convex) membrane curvature, consistent with a function of endophilin early during invagination. Insertion of aminophospholipids into the plasma membrane by a transmembrane lipid transporter resulted in an enhancement of endocytosis [122].

It was originally proposed that the LPAAT activity would lead to an increasing negative (concave) curvature and constriction of the neck of the coated pit [88]. This would however only increase the cross-section area of the coated pit. Alternatively, PA might undergo transbilayer movement as seen in model membranes [123]. Finally, PA might induce a deep penetration of dynamin into the membrane of the neck region facilitating the fission reaction (unpublished observations in [72]).

The cortical actin present at the plasma membrane might act as a barrier for the invagination of the coated membrane (Fig. 5). Several accessory proteins could be required to regulate the tension of the plasma membrane caused by cortical actin. The role of filamentous actin (F-actin) and cortical actin in endocytosis remains controversial. Membrane-permeant toxins which either stabilize or disrupt F-actin, inhibit endocytosis in some cell types [124-126] while they have little effect in others [127-129]. A difference in the cortical actin between different cells could be the reason for this discrepancy [129]. F-actin is localized to non-release-sites (i.e. non-active zones) in the frog neuromuscular junction [130]. Disruption of the actin cytoskeleton was found to increase the mobility of coated pits in the plasma membrane of non-neuronal cells [111]. Injection with synaptojanin-perturbing reagents in lamprey synapses (**Paper IV**), induced a filamentous matrix in areas of endocytosis. Similar cytoskeletal filaments were observed after phalloidin injection (Shupliakov et al., manuscript in preparation), which indicates that these filaments consist of F-actin. Genetic studies of synaptojanin null mutants also reported an induction of a cytoskeletal matrix [86,87]. Synaptojanin could potentially affect the rearrangement of the actin cytoskeleton [131] by dephosphorylating $PI(4,5)P_2$, which binds to many actin-regulating proteins, and thus decrease the actin-mediated tension of the plasma membrane. Both synaptojanin and dynamin interact with the SH3-domain proteins syndapin I and II, that also binds to N-WASP [59,132], a stimulator of Arp2/3 induced actin filament nucleation. Overexpression of full-length syndapins in neuro-endocrine cells, had a strong effect on cortical actin organization [132]. Profilin, which is a potent regulator of actin dynamics, also binds to dynamin [133]. Dynamin is present on early stages of coated pits (**Paper V**; [49]) and antibody-mediated disruption of the dynamin function inhibited clathrin coat formation and perhaps also the invagination of the coated membrane (**Paper V**). In non-neuronal cells, overexpression of dynamin with a mutation in the GTPase domain (K44A) increased the amount of transferrin-receptors on the cell surface [47,49]. Dynamin 2aa immunoreactivity is co-localized with filamentous actin at podosomes and overexpression of mutant dynamin abolished the podosomes [134]. Also, clathrin coated pits seem to form on areas of the plasma membrane devoid of actin filaments [129]. One possible function of dynamin could be to regulate the cortical actin to allow clathrin-mediated budding from the plasma membrane (Fig. 5).

The onset of synaptic vesicle endocytosis

A number of studies have investigated the early events in clathrin-mediated endocytosis and the factors required to selectively internalize the correct patch of membrane. In the nerve terminal this seems to be even more important since

the rate of synaptic vesicle retrieval must be very fast to keep up with high rates of vesicle release. Moreover, the recycling synaptic vesicle proteins must be separated and sorted from other transmembrane proteins in the plasma membrane. It has been suggested that the Ca^{2+} influx during vesicle release is essential to trigger synaptic vesicle retrieval, and that endocytosis and exocytosis are coupled by a common Ca^{2+}-signal (see Introduction). This hypothesis was tested in **Paper I** by temporally dissociating Ca^{2+} mediated exocytosis from endocytosis. The results show that clathrin-mediated synaptic vesicle endocytosis can be initiated simply by adding extracellular Ca^{2+}, without additional action potential mediated Ca^{2+} influx. Micromolar concentrations of extracellular Ca^{2+}, well below the concentrations normally present, were sufficient to induce clathrin coat formation. This suggests that it is not necessary to elevate the intracellular Ca^{2+}-level above the resting level in order to initiate endocytosis. Thus it seems likely that exo- and endocytosis are not coupled by a common Ca^{2+}-signal. The data also indicate that the synaptic vesicle membrane added to the plasma membrane serves as an adequate signal to trigger synaptic vesicle endocytosis. If there would be a strict coupling between exo- and endocytosis by a Ca^{2+}-signal, inhibition of exocytosis without reduced Ca^{2+} entry (e. g. after inhibition by neurotoxins), would trigger endocytosis although no vesicle membrane had been added to the plasma membrane. As a consequence, the nerve terminal would be reduced in size. However, Ca^{2+} may still have a role in modulating the rate of endocytosis. Other studies have shown that elevation of the Ca^{2+}-concentration above the resting level by Ca^{2+}-influx, may increase the speed of secretory vesicle endocytosis [13,95]. The mechanism could, for instance, be an activation and binding of the endocytic protein complex via calcineurin dependent dephosphorylation of dynamin, amphiphysin and synaptojanin (Fig. 5; [40]).

Dynamin-mediated fission

Many studies have implicated dynamin as a key component in the fission of the coated pit from the plasma membrane (see Introduction). Microinjection of compounds that disrupt the interaction between dynamin and amphiphysin (**Paper II**) caused an accumulation of deeply invaginated coated pits with constricted necks. Similar, although not identical, results were obtained after perturbing the dynamin-endophilin interaction (**Paper IV**). This provides strong evidence that dynamin is involved in fission of clathrin-coated pits, which is consistent with studies in both *Drosophila* neurons and mammalian cells (see Introduction). It also indicates that amphiphysin and endophilin participate in fission and that the interactions between these three proteins are functionally important for the budding (Fig. 5). This is consistent with the results obtained in other studies. Overexpression of the SH3-domain of amphiphysin and endophilin, inhibited clathrin-mediated endocytosis [88,135,136]. Primarily, later stages of coated intermediates were inhibited [136], which agrees with a role of these protein-protein interactions in fission.

Electron-dense collars (or rings) have been observed around the neck of endocytic buds, even in unperturbed synapses (Table 1; **Paper I**), but their relevance for the fission of coated pits remains controversial. Two types of constricted endocytic buds were observed in the dynamin *shibire* mutant: uncoated buds with collars and clathrin-coated buds that did not contain a collar [9,43,46]. Endocytic intermediates both coated and collared were only occasionally observed. Dynamin formed ring-like structures on synaptosomal membranes incubated with GTPγS [50] but only a portion of the dynamin-coated tubules were covered with clathrin-coats. In addition, amphiphysin is a component of the dynamin-coated tubules [39], and may even by itself form ring-like structures around lipid membranes *in vitro* [68]. Endophilin is also localized to dyna-

min-coated tubules generated *in vitro* and is required for the formation of these tubules (**Paper III**). Injection of GSTamphSH3 and PP15 into intact lamprey terminals (**Paper II**), caused an accumulation of mostly uncollared coated pits, whereas injection of two other reagents, GSTendoSH3 or GTPγS (**Paper IV**), induced coated pits with electron-dense rings and spirals. These experiments demonstrate that electron-dense collars exist *in vivo* and that they are indeed closely associated with clathrin coats. The ring-like structures observed in the microinjection experiments (**Paper IV**), on synaptosomal membranes [50] and artificial liposomes [36,52,63,68], might not be the same structures since they are different with respect to density, regularity and inter-ring spacing. The morphology of ring-like structures induced by either dynamin or amphiphysin separately or those induced in the presence of both proteins, has been found to differ [68]. The microinjection experiments in **Papers II** and **IV** also suggests that the amphiphysin-dynamin interaction can be temporally separated from the dynamin-endophilin interaction. Although the role of the electron-dense collars is still unclear, dynamin, amphiphysin and endophilin seem to be functional partners in a fission machinery.

Coated vesicle formation is unidirectional

Synaptic vesicles undergo multiple rounds of fusion with the presynaptic membrane, incorporation into a coated pit and subsequent coat removal. To prevent this process from going in the reverse direction, a strict unidirectionality must be imparted into the mechanism.There are several possible ways this can be accomplished. One way would be to regulate the activity of individual components and the protein-protein interactions by e. g. a series of protein phosphorylation and dephosphorylation reactions at distinct stages [38-40,96].Another way to accomplish this would be to have a cycle of phospholipid modifications in parallel with the synaptic vesicle cycling (Fig. 5). There is compelling evidence that the PIP-phosphatase synaptojanin is involved in this mechanism. Microinjection of the synaptojanin-derived peptide PP19 and antibodies against synaptojanin (**Paper IV**) as well as mutations in the synaptojanin gene [86,87], led to an accumulation of free coated vesicles. Interestingly, the coated vesicles were observed at great distances from the synaptic site, which suggests that they were lost from the endocytic recycling machinery. Although clathrin coated vesicles can be purified from brain tissue [137], it is unclear if coated vesicles with a complete clathrin coat exist *in vivo* during unperturbed synaptic vesicle recycling. Coated vesicles are usually not observed in intact unperturbed lamprey nerve terminals. Moreover, it is unclear whether the coated vesicles that were observed in other ultrastructural studies [1,138] are connected to the plasma membrane or not. In **Paper IV**, the coated vesicles were examined on serial sections and on thicker sections using intermediate voltage electron microscopy to be absolutely sure that they were not connected to any other membranes. Short-lived clathrin-coated structures were detected by monitoring flourescently-tagged clathrin heavy chain in COS-1 cells [111], which is consist with a rapid uncoating after the fission reaction.

One likely explanation for the presence of coated vesicles after synaptojanin disruption, is that synaptojanin facilitates the shredding of the clathrin coat by dephosphorylating PIPs in the vesicle membrane (Fig. 5). This would weaken coat-lipid interactions and promote ATP-dependent uncoating, which is mediated by the Hsc70/auxilin complex [139,140]. If so, at least one PIP-kinase should be active before or during the clathrin-coat formation to phosphorylate PIP to $PI(4,5)P_2$ in the vesicle membrane to promote coat binding (Fig. 5).

Results from **Paper IV** show that the SH3-domain of endophilin is involved in both dynamin-mediated scission of the coated pit and in synaptojanin-mediated uncoating of the free vesicle. This suggests that endophilin may act as a switch between the two processes to prevent a premature uncoating of the coated pit when it is still connected with the membrane. A cou-

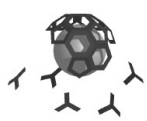
pling of fission and uncoating would allow for a rapid stripping of the clathrin coat directly after fission. This would allow a strict vectoriality of synaptic vesicle endocytosis. In this scenario, dynamin would interact with endophilin during fission, but as soon as the free vesicle is formed, the SH3-domain endophilin would become available to interact with synaptojanin (Fig. 5). Synaptojanin may either be present at the coated pit during the coat formation or be recruited from a cytosolic pool after fission (**Paper IV**).

Endocytic proteins may have multiple actions in endocytosis

For many of the injected compounds, there was accumulation of one morphologically distinct type of coated intermediate, probably representing an inhibition of a specific step of coated vesicle formation. However, many of the reagents probably affected additional steps in the synaptic vesicle cycle, which could be masked by the inhibition of an earlier step. This seems plausible since all the studied proteins have multiple binding sites to other endocytic proteins (see Introduction). Several observations made in this study also favor this idea. Two types of coated intermediates, deeply invaginated coated pits and free coated vesicles, accumulated in PP19 injected synapses (**Paper IV**). This was interpreted as a kinetic delay of the fission reaction so that free coated vesicles could form. On the other hand, disrupting the same interaction with GSTendoSH3 led to an accumulation of constricted coated pits with electron dense rings while free coated pits were not present. One explanation for this discrepancy could be a stronger inhibition of the fission step by GSTendoSH3. The relatively small number of free coated vesicles together with expansions of the plasma membrane after synaptojanin antibody injection, could be interpreted as a block early in endocytosis that prevented the formation of coated vesicles (**Paper IV**). With the endophilin antibodies, the strong inhibition at the shallow coated pit stage appear to be bypassed through invaginations of the plasma membrane so that coated intermediates of later stages could form. Finally, the inhibition of clathrin coat formation by dynamin antibody injection (**Paper V**) probably masked the more established role of dynamin at the fission step (**Paper II** and **IV**).

In general, there seemed to be a block at earlier stages in endocytosis after injection of antibodies against endophilin (**Paper III**), synaptojanin (**Paper IV**) and dynamin (**Paper V**), as compared the injections of reagents directed to SH3-domain interactions (**Papers II** and **IV**), in which later stages of coated intermediates accumulated. If one assumes that each of these endocytic proteins have functions at multiple steps in endocytosis, such a difference would be expected. The antibody injection probably affected the entire protein and several of its functions might have been impaired. As indicated above, inhibition of early stages might mask the inhibition of later stages. On the other hand, injection of SH3-domain directed reagents perturbed only specific interactions. The difference in size between the relatively large antibodies and the smaller fusion proteins or peptides, could also contribute to this difference. This is consistent with another study in permabilized HeLa cells, where the authors used a biochemical assay that permitted a separation of clathrin-mediated endocytosis into early and late stages [136]. Overexpression of the SH3-domains from several different endocytic proteins preferentially led to an inhibition of later stages of endocytosis [136].

The complexity of vesicle retrieval at the plasma membrane does not only lie in the great number of accessory endocytic proteins participating in the process, but also in the fact that each protein have several domains. Each endocytic protein may thus have several functions at multiple steps of the process. The present work have revealed the function of some of the protein domains at distinct stages of endocytosis.



Conclusions

1. Impairment of the clathrin-mediated endocytic machinery in a living synapse inhibits synaptic vesicle endocytosis.
This provides strong evidence that the main pathway for synaptic vesicle retrieval is through clathrin-mediated endocytosis.

2. Exo- and endocytosis can be temporally separated by tonic action potential stimulation followed by removal of extracellular Ca^{2+}. Synaptic vesicle retrieval can be initiated by readding a low micromolar concentration of extracellular Ca^{2+}, without additional action potential stimulation.
The results indicate that the synaptic vesicle membrane incorporated in the plasma membrane during exocytosis, rather than action potential-evoked Ca^{2+}-influx, is a sufficient trigger of synaptic vesicle endocytosis.

3. Antibody-mediated disruption of the function of endophilin leads to a massive accumulation of shallow clathrin-coated pits on the presynaptic plasma membrane.
The results suggest that endophilin is required for the invagination of the clathrin-coated pit in the transition from early to late stages of synaptic vesicle endocytosis.

4. Microinjection of compounds that disrupt the SH3-domain interaction between amphiphysin and dynamin blocks clathrin-mediated retrieval at the stage of deeply invaginated, constricted coated pits. Disrupting another SH3-domain interaction between dynamin and endophilin results in an inhibition at a similar stage, although electron-dense collars surround the neck of the coated pits.
These data indicate that SH3-domain interactions have an essential role in endocytosis and that dynamin and its interaction with amphiphysin and endophilin participate in the fission of the neck of the coated pit.

5. Injection of a peptide blocking the endophilin-synaptojanin interaction and antibodies to the proline-rich domain of synaptojanin induce accumulation of free clathrin-coated vesicles.The same peptide inhibits the binding of synaptojanin to liposomes *in vitro*.
These results indicate that synaptojanin is recruited to the free coated vesicle by the interaction with endophilin to faciliate the removal of the clathrin coat.They also suggest that the SH3 domain of endophilin may act as part of a molecular switch that couples the fission reaction to uncoating and imparts a strict vectoriality to synaptic vesicle endocytosis.

6. Antibody-mediated disruption of dynamin blocks synaptic vesicle endocytosis at a stage preceding clathrin coat formation.
These results suggest that dynamin participates at early stages of clathrin-mediated endocytosis in addition to its established role in fission. Several mechanisms are plausible, for example a regulation of the cortical actin by recruiting additional proteins, e. g. profilin.This would allow clathrin coat polymerization on the plasma membrane devoid of cortical actin filaments.

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