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MOLECULAR MECHANISMS CONTROLLING DYNAMIN RECRUITMENT TO SITES OF ENDOCYTOSIS BY SH3-DOMAIN CONTAINING PROTEINS

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Cover image shows clathrin-coated pits in a synapse injected with GTPγS, stimulated at 5 Hz and labelled with gold-conjugated antibodies against dynamin.

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ABSTRACT

Endocytosis is a general cellular mechanism, which regulates a number of important events, including recycling of synaptic vesicles. Clathrin-mediated endocytosis is the best-characterized endocytic pathway in the cell. This internalization process involves the coat protein clathrin, the adaptor protein AP2 and a number of accessory proteins. All endocytic events involve a fission step, when the membrane is severed. Fission during clathrin-mediated endocytosis is regulated by the large GTPase dynamin. Dynamin self-assembles into spirals on lipid membranes and is believed to functions as a mechanochemical enzyme, severing the membrane by force generated by a GTP hydrolysis-dependent conformational change in the spiral structure.

Clathrin-mediated endocytosis is the main pathway to recycle synaptic vesicles after exocytosis in the nerve terminal. Synaptic vesicle recycling is essential for sustained neurotransmission. It occurs in the periactive zone of nerve terminals. Several endocytic proteins are known to migrate from the synaptic vesicle cluster, where they reside at rest, to the periactive zone upon stimulation and Ca^{2+} entry. Dynamin is dependent on interactions with SH3-domain containing proteins to localize to sites of endocytis in non-neuronal cells. In the nerve terminal, dynamin interacts with a number of SH3-domain containing proteins, but the exact roles of these interactions are not known.

In this thesis, we investigate how the dynamin-binding proteins intersectin, endophilin and syndapin regulate the recruitment of dynamin to the periactive zone and the function of dynamin during fission to recycle synaptic vesicles.

We show that dynamin co-localizes with intersectin and endophilin in the synaptic vesicle cluster at rest and at clathrin-coated pits in the periactive zone in stimulated nerve terminals. Intersectin is important to regulate the amount of dynamin that is recruited to the periactive zone and to scaffold the endocytic process via the interaction with the α- and β-appendages of the clathrin adaptor protein AP2.

The AP2-intersectin interaction regulates the ability of intersectin to bind synaptojanin, which promotes uncoating of synaptic vesicles after they have been severed from the plasma membrane.

Intersectin and endophilin are required for proper dynamin organization at necks of ccps during clathrin-mediated endocytosis. Endophilin and dynamin assemble into a complex on membrane necks, which is required for fission.

Intra-molecular interaction between the SH3- and the F-BAR-domain of syndapin regulates its membrane tubulation activity *in vivo* and *in vitro.* This auto-inhibition is disrupted by interaction with dynamin.

We propose that intersectin, endophilin and syndapin participate in the synaptic vesicle recycling process by regulating the amount of dynamin that is recruited to the periactive zone, by targeting dynamin to necks of ccps and by mediating the assembly of a pre-fission complex, which regulates membrane scission.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will are referred to in the text by their roman numerals.

I. Evergren E.[*], Gad H.[*], Walther K.[*], Sundborger A., Tomilin N., Shupliakov O. (2007) **Intersectin is a negative regulator of dynamin recruitment to the synaptic endocytic zone in the central synapse**, J Neurosci. 27(2).
[*]*Shared authorship*

II. Pechstein A., Bacetic J.[*], Vahedi-Faridi A.[*], Gromova K.[*], Sundborger A.[*], Tomilin N., Krainer G., Vorontsova O., Schäfer J., Owe S., Cousin M., Saenger W., Shupliakov O., Hauke V. (2010) **Regulation of synaptic vesicle recycling by complex formation between intersectin 1 and the clathrin adaptor complex AP-2**, PNAS, 107(9). [*]*Shared authorship*

III. Rao Y.[*], Ma Q.[*], Vahedi-Faridi A., Sundborger A., Pechstein A., Puchkov D., Shupliakov O., Saenger W., Hauke V. (2010) **Molecular basis for SH3-domain mediated regulation of F-BAR-mediated membrane deformation**, PNAS, 107(18). [*]*Shared authorship*

IV. Sundborger A., Soderblom C., Vorontsova O., Evergren E., Hinshaw J., Shupliakov O. (2010) **An endophilin-dynamin complex promotes budding of clathrin-coated vesicles during synaptic vesicle recycling**, Reviewed and re-submitted to J Cell Sci.

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LIST OF ABBREVIATIONS

Ccp	clathrin-coated pit
Ccv	clathrin-coated vesicle
PS	Phosphatidylserine
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
SH3	Scr homology 3
PH	Pleckstrin homology
GED	GTPase effector domain
PRD	Proline-rich domain
NMJ	Neuromuscular junction
EM	Electron microscopy
CGN	Cerebral Granule Neuron
GTP	Guanosine-5’-triphosphate
GTPγS	Guanosine 5'-O-[γ-thio]triphosphate
Eps15	Epidermal growth factor receptor substrate 15
Arp2/3	Actin related proteins 2, and 3
ENTH	Epsin N-terminal homology
AP180	Adaptor protein 180 kDa
EH	Eps15 homology
Dap160	Dynamin associated protein 160 kDa
BAR	BIN/Amphiphysin/Rvs161/167
GFP	Green Fluorescence Protein
TIRF-M	Total Internal Reflection Fluorescence Microscopy
EPSP	Excitatory Postsynaptic Potential
NPF	Asn-Pro-Phe
SNX9	Sorting nexin 9
VAMP	Vesicle-Associated Membrane Protein
Sjl2	Synaptojanin-like protein 2

1 INTRODUCTION

1.1 ENDOCYTOSIS

Endocytosis in a general cellular mechanism, utilized for a wide variety of functions, including internalization of extracellular hormones and cell-surface receptors, nutrient uptake, phagocytosis, antigen-presentation, cell migration, mitosis and recycling of synaptic vesicles[1-3]. It is also known that many pathogens, such as the HIV virus and *Listeria* hi-jack endocytic processes to gain entry into the cell[4, 5]. There are various endocytic pathways in the cell (Fig. 1).

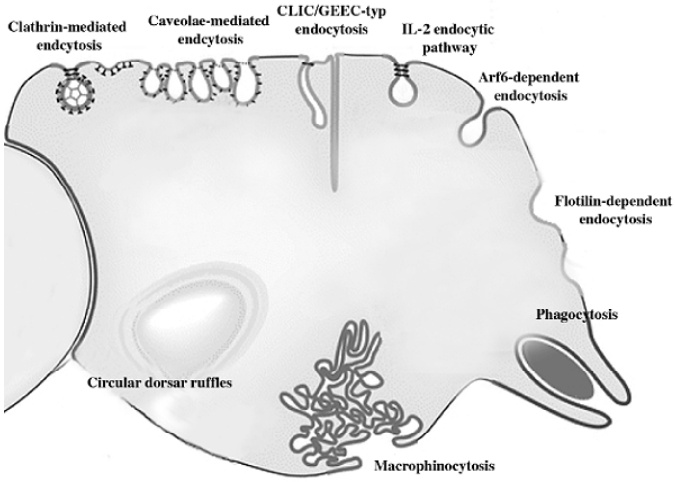

Figure 1. Schematic illustration of different endocytic processes in the cell. The GTPase dynamin is required to mediate plasma membrane fission during some of these endocytic events.
Adapted from [1]

These may be classified according to the requirement of coat proteins, e.g. clathrin-, or caveolin-mediated endocytosis; the type of cargo internalized, e.g. IL-2R; or the function of the internalization, e.g. macropincytosis. Clathrin-mediated endocytosis is the best characterized internalization process in the cell. It entails the formation, maturation and scission of clathrin-coated pits (ccps), loaded with cargo, from the plasma membrane and results in vesicles that are further transported to different intracellular compartments (Fig. 2).

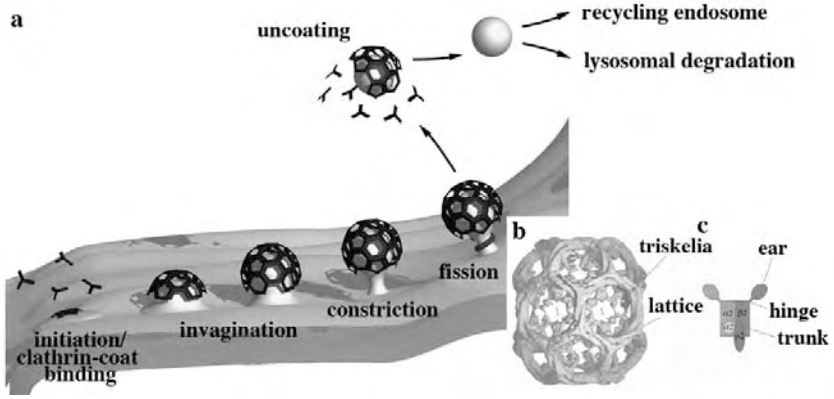

Figure 2. Clathrin-mediated endocytosis. a) The progression of clathrin-mediated endocytosis: recruitment of coat proteins, membrane invagination and constriction, dynamin-mediated fission and uncoating. b) The structure of the coat protein clathrin. Clathrin triskelia assembled from three heavy chains and three light chains of clathrin polymerize into lattices. c) The adaptor protein AP2 is composed of four subunits. The appendages, or ears of AP2 are connected to the trunk via flexible hinges.
Adapted from [6, 7]

1.2 CLATHRIN-MEDIATED ENDOCYTOSIS

1.2.1 The clathrin adaptor protein AP2

Clathrin-mediated endocytosis involves an intricate web of interactions between the clathrin coat machinery, endocytic accessory proteins, such as dynamin, endophilin, intersectin, epsin and synaptojanin and components of the plasma membrane (Fig. 2). Clathrin-mediated endocytosis is initiated by recruitment and assembly of clathrin triskelia. Each clathrin triskelia is composed of three clathrin heavy chains and three clathrin light chains, which assemble into lattices at the plasma membrane, as shown in figure 2. This is promoted by the clathrin adaptor protein AP2 (Fig. 2c)[8, 9]. AP2 is involved in clathrin-mediated endocytosis at the plasma membrane[8, 9], while other adaptors, such as AP1 and GGA1 are involved in clathrin-mediated membrane trafficking events in ER-Golgi and endosomal compartments[10, 11]. AP2 is a hetero-tetrameric protein complex composed of four subunits, α2, β2, μ2 and σ2[11]. It coordinates recruitment and polymerization of clathrin with binding and sorting of cargo, such as surface-bound receptors. Cargo binding and sorting is mediated predominately by the AP2 μ2 subunit by recognition of specific peptide motifs on the cargo, (YxxF and [D/E]xxxL[L/I])[8, 12-15]. AP2 may also bind a large number of endocytic proteins via its two extended appendages, or ears[8, 16]. The ears are connected to the trunk via flexible hinges. Both ears contain two putative binding sites for endocytic proteins, the platform subdomain (top site) and the β-sandwich subdomain (side site). These sites recognize a series of different peptide motifs[8, 16] and *in vitro* binding studies show that the α2-ear interacts with amphiphysin[17], synaptojanin[18], epsin, intersectin, stonin and NECAP[18-21], while the β2-ear has been suggested to interact with eps15, AP180, amphiphysin, sorting nexin 9 (SNX9), epsin, clathrin[13] and synaptojanin[8, 14, 18]. It was suggested that during clathrin-mediated endocytosis, several endocytic proteins bind the appendages of AP2 simultaneously, creating a network of clustered proteins at the site of clathrin assembly. This indicates that AP2 functions as a major coordinator of clathrin-mediated endocytosis[11]. Knockdown of AP2 is lethal in *Caenorhabditis elegans* (*C.elegans*), *Drosophila* and mice[22-24] and RNAi, which acutely perturbs the protein in cells causes an accumulation of ccps[25] and mis-localization of endocytic proteins[16, 18, 26, 27].

1.2.2 $PI(4,5)P_2$

AP2 can be recruited to the plasma membrane by interactions with the membrane component $PI(4,5)P_2$[13]. This interaction is mediated by the μ2 and β2 subunits of AP2 and mutations in either are known to cause mis-localization of AP2 and a subsequent arrest in clathrin-mediated endocytosis[13]. Also, binding of cargo to the μ2 subunit is permitted only after $PI(4,5)P_2$ has bound to the β2-ear, which causes a conformational change in the protein complex, exposing the cargo binding site[13]. Apart from binding and clustering AP2 at the plasma membrane, $PI(4,5)P_2$ also interacts with many other endocytic proteins, such as epsin, AP180, dynamin, profilin, syndapin and N-WASP[28-30]. $PI(4,5)P_2$ is generated from PI(4)P by the phosphoinositol phosphate kinase 5 (PIPK5)[28, 31]. Knockdown of PIPK5γ66, the neuronal-specific isoform of PIPK5[32] caused sever effects on synaptic transmission, attributed to failure in synaptic vesicle

recycling due to the loss of $PI(4,5)P_2$ in the plasma membrane[29]. Interestingly, in nerve terminals, $PI(4,5)P_2$ production is promoted by the interaction between PIPK5γ66 and AP2[33]. The interaction is regulated by a depolarization-dependent dephosphorylation of PIPK5γ66 by calcineurin, a phosphatase implicated in regulation of clathrin-mediated endocytosis in the nerve terminal[33]. This provides a functional link between depolarization-induced fusion of synaptic vesicles and initiation of synaptic membrane recycling[33]. Regulation of $PI(4,5)P_2$ is important throughout the clathrin-mediated endocytosis process – to initiate clathrin coat assembly, as just described; to mediate membrane curvature and fission, which will be discussed later; and to strip newly formed vesicles from their clathrin coats[29, 34]. After fission, hydrolysis of $PI(4,5)P_2$ causes disassemble of AP2, clathrin and other coat components, releasing the vesicle from the coat in a process referred to as uncoating[13]. This process is regulated by the phosphoinositol phosphatase synaptojanin[29, 35, 36]. Synaptojanin knockdown in *C.elegans*[37], *Drosophila*[38] and mice[35, 39] strongly inhibits endocytosis, presumably due to a build-up of $PI(4,5)P_2$ in the plasma membrane[35]. Synaptojanin deletion mutants also exhibit a prolonged recovery time after synaptic depression, attributed to a defect in the recycling of synaptic vesicles[35, 38]. The role of synaptojanin in uncoating of synaptic vesicles was strengthened by the finding that acute perturbation of synaptojanin function in nerve terminals of *Lampetra fluviatilis* (lamprey) giant axons caused an accumulation of free clathrin-coated vesicles[36]. This suggests a crucial regulatory role of synaptojanin in clathrin-mediated endocytosis, to regulate the amount of $PI(4,5)P_2$ at the plasma membrane[29, 35, 36].

1.2.3 Membrane bending proteins

As clathrin polymerizes, the plasma membrane must invaginate to accommodate the rigid shape of the assembling clathrin lattice. Membrane curvature is the result of complex interplay between proteins, lipids and physical forces applied to the membrane[40]. Intrinsic properties of lipids in the membrane play a role, as they determine the elasticity and the rigidity of the membrane[41]. Some lipids are more prone to curve than others, cholesterol for example[41]. Extrinsic factors, such as proteins and the actin cytoskeleton, may also promote membrane curvature by different means[41-44]. During clathrin-mediated endocytosis, initial membrane curvature is promoted by AP2, along with epsin, another clathrin adaptor[45]. Members of the epsin protein family contain epsin N-terminal homology (ENTH) domains that interact with $PI(4,5)P_2$ in the plasma membrane[46, 47]. Overexpression of $PI(4,5)P_2$ binding deficient- or ENTH-deleted epsin mutants causes an inhibition of EGF receptor internalization, indicating that the ENTH domain has an important function in endocytosis[46, 47]. The ENTH domain is composed of α-helices and the crystal structure of the domain bound to $PI(4,5)P_2$ revealed that the N-terminal most helix, helix 0, forms a concave surface with positive charge, enabling binding to the negatively charged $PI(4,5)P_2$. Helix 0 is also suggested to form an amphiphatic helix (AH), which inserts into the membrane and further promotes membrane bending[46, 47]. Proteins containing Bin/Amphiphysin/Rvs161/167 (BAR)-domains comprise another group of proteins with membrane binding- and bending properties[48-51]. They are implicated in clathrin-mediated endocytosis to promote curvature and constriction of the clathrin-coated pit[52, 53]. Insight into the mechanism of their function in endocytosis and other membrane deforming events in the cell, was first suggested from the crystal structure of the BAR-domain of

amphiphysin[53]. Amphiphysin BAR-domains dimerize into a crescent, banana-shaped structure, as shown below in figure 3.

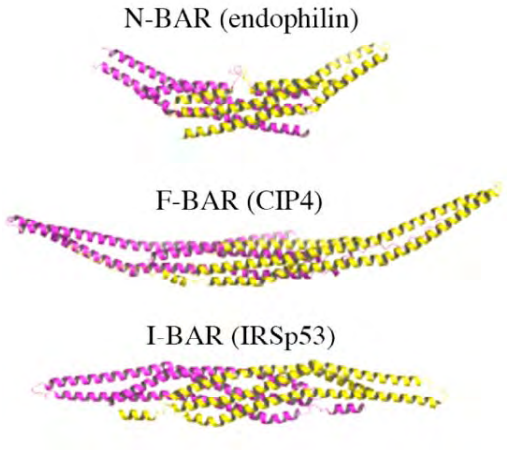

Figure 3. The common structure of all BAR-domains: three long α-helices (helix 1-3) connected via two loops. N-BARs also contain a forth helix (helix 0) in the N-terminal most part of their domains. Dimerization is mediated via hydrophobic interactions between the inner surfaces of the individual BAR-domain, creating crescent-shaped dimers.
Adapted from [1]

This finding lead to the suggestion that membrane curvature is obtained as the dimer binds and enforces its shape onto the membrane[50, 53-56]. This notion was further supported by the finding that *in vitro*, BAR-domain proteins promote formation of lipid tubes of different diameters, ranging from 20-200 nm, which corresponds to the shape of their respective BAR-domain dimers[53-55]. Extensive research in this field has shown that bending is also dependent on the positive charge that lines the concave surface of the BAR-domain dimer, enabling interactions with negatively charged lipids, such as $PI(4,5)P_2$, in the lipid membrane[49, 50]. Also, *in vitro*, different BAR-domain proteins bind to (and promote formation of) lipid tubes of different diameters,[49, 51, 53, 57] which suggests that different BAR-domain proteins preferentially bind membranes of certain curvatures, a concept referred to as curvature-sensing[53]. BAR-domains are classified into classical BARs, F-BARs and just recently, I-BARs, to further distinguish the specific properties. Classical BAR-domains that contain an AH at the very N-terminal part of the domain are referred to as N-BARs[53]. The N-BAR-domain proteins endophilin and amphiphysin promote formation of narrow lipid tubes, resembling constricted necks of ccps, further supporting their involvement in clathrin-mediated endocytosis[51-53, 58]. The AH of N-BAR-domain dimers inserts into the membrane, which is suggested to mediate the actual lipid binding, while the BAR-domain dimer mediates the bending[57, 59-61]. It was recently suggested that the ability of proteins to insert hydrophobic residues into membranes, such as AHs, may be a general mechanism of membrane binding, not exclusive to BAR-domain proteins[59-61]. The adaptor protein epsin is one such protein. F-BAR-domains dimerize into structures with a slightly larger radius of curvature than BAR-domains (Fig. 3) The larger curvature suggests that F-BAR-domain proteins are involved in membrane bending events that promote less curvature, or in the process of clathrin-mediated endocytosis, at an earlier stage of pit formation[50, 51, 53, 55, 57]. Membranes are more or less rigid, and to induce curvature, the rigidity of membrane-interacting proteins must exceed that of the membrane[40, 62]. The F-BAR-domain of CIP4 has been shown to oligomerize into a rigid coat structure on the membrane, which may be crucial to stabilize membrane curvature[55]. I-BAR-domain dimers exhibit the reverse, convex curvature and have recently been identified in the formation of membrane protrusions, e.g. in filopodia formation[63].

1.2.4 Actin

Actin may also be involved in membrane bending[64-66]. In yeast, actin polymerization is responsible for initiating membrane curvature during endocytosis[64-66]. FRAP experiments show that actin filaments anchor to the membrane bud and further actin polymerization is though to provide force enough to pull the budding membrane, thus creating curvature[64, 66, 67]. This is proposed to recruit curvature-sensing BAR-domain proteins, which promotes further membrane bending, which recruits more BAR-domain proteins, thus creating a positive feed back loop to cluster BAR-domain proteins at constricted membrane necks. This process is directly coupled to fission in yeast, as the concentration of BAR-domain proteins at the neck shields the clustered $PI(4,5)P_2$ from the action of the synaptojanin orthologue sjl2 (synaptojanin-like protein 2). Sjl2, once recruited to the membrane bud, hydrolyzes $PI(4,5)P_2$ at the coat only, creating a phase separation in the membrane at the coat-neck interface. This causes the membrane to destabilize and undergo fission[64, 68]. Thus, actin is essential to promote membrane curvature and fission in yeast[64-66, 69].

1.2.5 Fission

Fission events in higher eukaryotic organisms are more diverse than in yeast. Fission during clathrin-mediated endocytosis requires the action of a large GTPase called dynamin to sever the plasma membrane[70, 71]. Many fission events are independent of coat proteins such as clathrin, or caveolin, but still require dynamin, though some fission events, such as intake of large particles via macropinocytosis and shiga toxin internalization, are independent of dynamin as well (see Fig. 1)[1, 72]. In these events, cholesterol and actin may play major roles[73, 74]. In yeast, there is no dynamin counterpart involved in endocytosis[64] and thus the membrane scission event is fundamentally different from fission during clathrin-mediated endocytosis. As of yet, there is no strong evidence for a direct role of actin in dynamin-mediated fission or even in clathrin-mediated endocytosis, but there are implications. A large number of dynamin-binding proteins interact with actin and either directly (cortactin, profilin), or indirectly (syndapin, intersectin) promote actin polymerization[75]. The actin-associated protein N-WASP, which interacts with the endocytic proteins syndapin and intersectin is recruited to sites of dynamin oligomerization[76]. Blocking of actin polymerization inhibits dynamin-mediated scission of ccps in 3T3 cells[77] and synaptic vesicle recycling in lamprey giant synapses[78], which is predominately dependent on clathrin-mediated endocytosis[2, 79-81]. The exact role of actin in this process remains obscure, though it has been suggested to participate in fission by providing force to pull on the budding membrane, as in yeast and by providing the means of recruitment of endocytic components to and from sites of endocytosis[75]. In synaptic nerve terminals, clathrin-mediated endocytosis used to recycle synaptic vesicles occurs is specialized areas, lateral to sites of exocytosis. Actin has been suggested to scaffold this endocytic process and to mediate transport of newly formed synaptic vesicles back to the active zone for consecutive rounds of release[78, 82, 83].

1.2.6 Dynamin

How dynamin mediates fission is still a matter of debate and will be discussed in some detail below. Dynamin was initially identified as an important protein in synaptic transmission from studies on the temperature-sensitive mutant of the *Drosophila* dynamin orthologue *shibire*, which revealed that the flies became paralyzed at non-permissive temperature[84]. The effect was later linked to the role of dynamin in fission during synaptic vesicle recycling[85] and since then, the requirement of dynamin has been established in many endocytic pathways[1]. There are three isoforms of dynamin, the neuronal specific dynamin 1[86], the ubiquitously expressed dynamin 2[87] and dynamin 3, which is found in brain, lungs and testis[88]. Different localization patterns of the isoforms have further suggested that they participate in fission at different localizations in the cell[89]. Dynamin 1 knockout mice survive for up to 15 days, indicating that dynamin 1 is not essential during development of the nervous system[90]. Synaptic transmission is not blocked in primary neuron cultures from these mice, but the evoked response is impaired[90]. These mice have dramatically decreased levels of total dynamin, but the levels of both dynamin 2 and 3 are normal, suggesting that there is also some overlap in the function of the various isoforms[90].

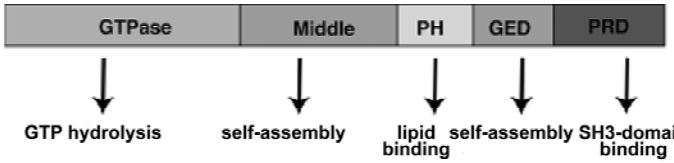

Figure 4. Schematic illustration of the domain structure of dynamin. Dynamin contains a GTPase domain, a middle domain, a PH-domain, a GED and a PRD.

As shown in figure 4, dynamin contains five distinct domains; a GTPase domain that binds and hydrolyzes GTP, a Pleckstin Homology (PH) domain, that enables binding to $PI(4,5)P_2$ in the plasma membrane, a GTPase effector domain (GED) that mediates self-assembly of dynamin into oligomers and facilitates GTPase activitiy, a middle domain, that has been suggested to contribute to self-assembly[91] and a proline-rich domain (PRD) that interacts with Scr homology 3 (SH3) domains of various proteins[92]. GTPase activitiy is promoted by binding to negatively charged lipids, such as PS and $PI(4,5)P_2$[93], self-assembly[94, 95] and protein binding to the PRD[93, 96]. In addition, the purified GED fragment can bind the GTPase domain directly, to promote GTPase activity[97], indicating that dynamin-mediated fission is a highly regulated event. Dynamin was first implicated in fission during clathrin-mediated endocytosis in mammalian cells after it was shown that overexpression of dynamin mutants block transferrin uptake (a marker for clathrin-mediated endocytosis) at the stage of membrane fission[98]. Dynamin was localized to ccps in these cells[98] and has also been detected on clathrin-coated membrane tubes formed in synaptosomes after treatment with GTPγS[99, 100]. It was shown early that the GTPase activitiy of dynamin is necessary for clathrin-mediated endocytosis[86, 98], though the exact mechanism of how this relates to fission was suggested when dynamin was shown to assemble into spirals in solution[101, 102] and that these structures, assembled onto lipid templates, constrict upon addition of GTP[103]. This was further confirmed by 3D reconstructions of dynamin in its constricted- and non-constricted state in vitreous ice using cryo-EM[104-106]. Upon addition of GTP, the diameter of these spirals decreases from 50 to 40 nm, indicating that a constriction of the protein-lipid structure has occurred[103-105]. This finding, along with the observation that dynamin spirals assembled on nanotubes increase in pitch

upon addition of GTP, suggested that GTP hydrolysis causes a conformational change in assembled dynamin spirals[102, 104, 105, 107] and that dynamin works as a mechanochemical enzyme to promote membrane fission[108-110].

1.2.7 Models of dynamin-mediated membrane fission

Dynamin may mediate fission of highly constricted membrane necks either by force generated from constricting and kinking the dynamin spiral like a corkscrew (Fig. 5, illustrations 1)[111] or by pulling on the membrane as a poppase until it is severed (Fig. 5, illustrations 2)[112, 113]. An alternative model of fission suggests that dynamin functions more like a classical GTPase, recruiting an effector protein (Fig. 5, illustration 3) in its GTP-bound state that in turn severs the membrane[114]. Whether a mechanochemical enzyme or an up-stream regulator of fission, dynamin and more specifically the GTPase activity of dynamin is essential for the endocytic process[98, 112, 115, 116].

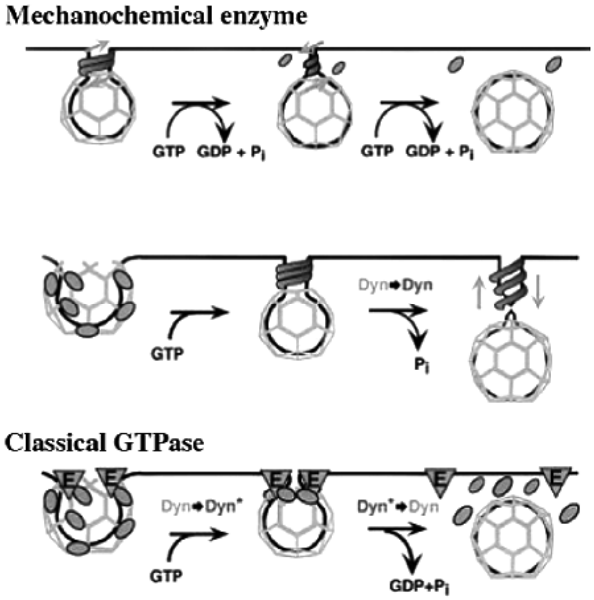

Figure 5. Schematic illustrations of dynamin-mediated membrane fission. Dynamin may function as a mechanochemical enzyme to sever the membrane neck by force, produced by GTP-dependent conformational change, or as a classical GTPase, which regulates fission by recruiting a down-stream fission protein in its GTP-bound state (Dyn*).
Adapted from [109]

It was recently shown using live imaging fission assays that dynamin may tubulate and sever PI(4,5)P_2-containing liposomes after addition of GTP, provided that sufficient lateral tension existed in the lipid tube[117]. In this study, tubes formed in the presence of dynamin underwent twisting and coiling. Addition of GTP contracted the tubes, indicative of an increase in tension, which proceeded tube fragmentation. This further supports the role of dynamin as a mechanochemical enzyme in fission. It was also shown that mere assembly of dynamin into oligomers is insufficient to deform lipid tubes with high tension, corresponding to that of the plasma membrane[118]. Dynamin (at a physiological concentration) required high membrane curvature to assemble on PI(4,5)P_2-containing templates[118]. This suggests that dynamin self-assembly and fission, occurs only if sufficient tension and curvature of PI(4,5)P_2–containing lipid membranes exists [93, 118].

To complicate matters, topical studies contradict the role of GTP hydrolysis and the conformational change-induced force as the cause of fission. Using real-time measurement of lipid tube fission similar to *Roux et al*, it was shown that GTP hydrolysis actually cause dynamin oligomers to disassemble[119, 120]. According to these studies, membrane fission is a result of several cycles of GTP-dependent squeezing

(upon binding) and relaxing (upon hydrolysis) of the tube, which promotes hemi-fission and thereby fission[91, 119-121]. It was further proposed that fission required that dynamin assembles in the presence of GTP. No fission was observed of pre-assembled dynamin tubes[120]. Also, short dynamin spirals were more efficient in severing the membrane, which casts doubt on previous fission assays analyzing the role of pre-assembled dynamin spirals.

Despite seemingly contradicting observations and theories, several circumstances dictate dynamin-mediated membrane fission: the presence of $PI(4,5)P_2$, membrane curvature, proper dynamin oligomerization and GTP binding- and hydrolysis.

1.2.8 Dynamin binding partners

It was previously shown that SH3-domains of several endocytic proteins severely impair clathrin-mediated endocytosis when overexpressed in cells[122]. There are a number of dynamin binding partners involved in clathrin-mediated endocytosis and all known binding partners of dynamin contain SH3-domains. Hence, it was proposed that dynamin is necessary but not sufficient to mediate fission *in vivo*[64, 122]. As mentioned previously, the critical GTPase activity of dynamin, is promoted by binding of SH3 proteins to the proline-rich domain of dynamin. This suggests a possible regulatory role of dynamin-binding proteins in fission.

SH3-domains are the best-characterized members of a growing family of protein-protein interaction modules[123]. They recognize proline-rich sequences in a large number of otherwise structurally and functionally different proteins[123]. There are an estimated 100 known interaction motifs available to SH3-domain containing proteins, which implicates them in many different, seemingly diverse functions in the cell, such as cytoskeletal rearrangement, cell movement-, and growth, cellular immune response, enzyme regulation and intracellular signalling cascades- and trafficking events[123, 124]. Class I ([R/K]xXPxXP) and class II (XPxXPx[R/K], 'X' is a non-glycine, hydrophobic residue while the 'x' denotes any amino acid) motifs of proline-rich domains bind to negatively charged pockets in SH3-domains[123-125]. These interactions have fairly low affinity and moderately low specificity. Several SH3-domains are often linked in tandem. This suggests that SH3-domain containing proteins are capable of mediating formation of large protein complexes with high rates of assembly- and disassembly.

Dynamin may bind $PI(4,5)P_2$ in the plasma membrane, but this is not sufficient to recruit dynamin to sites of endocytosis[126]. Dynamin mutants lacking the PRD fail to localize at the membrane[126]. This suggests that dynamin is recruited to ccps to perform fission by one of its SH3-domain binding partners[127]. This notion was suggested by studies of synaptic vesicle recycling in lamprey synapses. Injection of the amphiphysin SH3-domain resulted in an inhibition of clathrin-mediated endocytosis in nerve terminals, with a massive accumulation of constricted ccps, indicative of a block at the fission stage[128]. In addition to amphiphysin, dynamin interacts with a number of SH3-domain proteins involved in endocytosis, incluing endophilin, intersectin, syndapin, cortactin, profilin and SNX9. In this thesis, we have focused on endophilin, intersectin and syndapin.

Intersectin contains 2 epsin homology (EH) domains, a central coiled-coil domain and 5 tandem SH3-domains. In addition, a splice form of intersectin (intersectin-long), expressed in neurons, also contains a DH-, a PH- and a C2-domain, that enables

interactions with the actin network[129]. The large number of SH3-domains in intersectin has suggested that it functions as a scaffolding protein in *Drosophila* NMJs[130]. Intersectin binds several endocytic proteins *in vitro* (dynamin, synaptojanin, N-WASP, clathrin, AP2 and synapsin)[130-132] and has been shown to co-localize with clathrin, dynamin and endophilin at endocytic sites in *Drosophila* NMJs[132]. Intersectin has also been detected on ccps in Cos 7 cells[129] and on necks of invaginated caveolae[133]. Overexpression of both the SH3A-domain[122] and the full-length protein[134] results in a block of transferrin uptake in cells, possibly by sequestering one or several endocytic binding partners[122]. Intersectin has been shown to play a role in synaptic vesicle recycling, as loss-of-function mutants exhibit a decline in EPSP after stimulation[132], however, its exact role in endocytosis is not clear. Endophilin is another endocytic protein known to bind dynamin *in vitro*[135].

Endophilin is similar to amphiphysin in the sense that is contains both an SH3- and a N-BAR-domain,[53] and that it interacts with both synaptojanin and dynamin in vitro [36]. It has been shown to play a role in clathrin-mediated endocytosis to recycle synaptic vesicles in *C.elegans* and *Drosophila*, predominately as a potential recruiter of synaptojanin – the protein responsible for uncoating of newly formed vesicles[35, 37]. Perturbation of endophilin SH3-domain interactions in giant synapses of lamprey promotes accumulation of free clathrin-coated vesicles, strengthening it's link to synaptojanin and uncoating, but it also results in an accumulation of constricted ccps with long necks, suggesting a possible role of endophilin in dynamin-mediated fission[36]. The ability of endophilin to remodel membranes has suggested that it is needed to mediate the high curvature required in ccp formation[52, 56, 57]. Injections of antibodies against the BAR-domain of endophilin causes an accumulation of shallow ccps in lamprey giant synapse, indicating a block at an early stage of clathrin-mediated endocytosis, presumably due to failure of endophilin to promote constriction of the ccp necks[136].

Syndapin (also called pacsin) is yet another SH3-domain protein containing a BAR-domain (more specifically an F-BAR domain), that interacts with dynamin[137, 138]. It also contains two NPF (Asn-Pro-Phe) motifs, enabling it to interact with EH-domain containing proteins[139]. Syndapin was found to co-localize with dynamin and N-WASP[140], but interestingly, not clathrin in neurons[141]. The Syndapin-N-WASP interaction promotes actin polymerization via activation of the Arp2/3 complex, which was found to be crucial for transferrin uptake in cells[140]. A direct role of F-BAR-domain containing proteins in dynamin-mediated fission was suggested in studies of the F-BAR protein FBP17, which is very similar to syndapin and has the ability to cause tube formation when overexpressed in cells[142]. When FBP17 mutants that were unable to bind dynamin, were overexpressed, tubes persisted, attached to the plasma membrane, supposedly due to failure of dynamin to localize at the membrane to mediate scission[142]. Overexpression of the SH3-domain of syndapin causes a block of transferrin uptake at a late stage, further suggesting that the action of F-BAR-domain containing proteins is necessary for dynamin function in endocytosis[122, 142]. Recent studies have reported that syndapin does not participate in clathrin-mediated endocytosis, but in an alternative route to recycle synaptic vesicles, activity-dependent bulk endocytosis[143, 144]. Syndapin has been suggested to function as a link between membrane remodelling events, dynamin-mediated fission and the actin network during endocytosis[76, 137, 138, 140, 141, 145, 146].

2 THE SYNAPSE AS A MODEL SYSTEM TO STUDY MEMBRANE UPTAKE

2.1 MEMBRANE UPTAKE IN THE NERVE TERMINAL

In the synaptic nerve terminal, maintained neurotransmission is dependent on efficient recycling of synaptic vesicles after exocytosis, when they fuse with the plasma membrane and release their content of neurotransmitters into the synaptic cleft. The major mode to re-form synaptic vesicles is via clathrin-mediated endocytosis (Fig. 6b), though other routes are also suggested to play a role in this recycling process[2, 79-81]. Kiss-and-run has been put forward as a faster and more efficient recycling pathway (Fig. 6a). This model proposes that synaptic vesicles never fully fuse with the plasma membrane as they dock, but rather retract and re-enter the readily releasable vesicle pool directly after releasing their content [147], possibly though a transient fusion pore[148]. Bulk endocytosis has been suggested as a compensatory mechanism to recycle excess membrane following intense stimulation, when the clathrin-mediated endocytosis machinery fails to keep up with the rate of exocytosis (Fig. 6c)[149, 150]. Recent findings also suggest an initial bulk event occurring fast, before the onset of clathrin-mediated endocytosis during certain stimulation paradigms[116, 151].

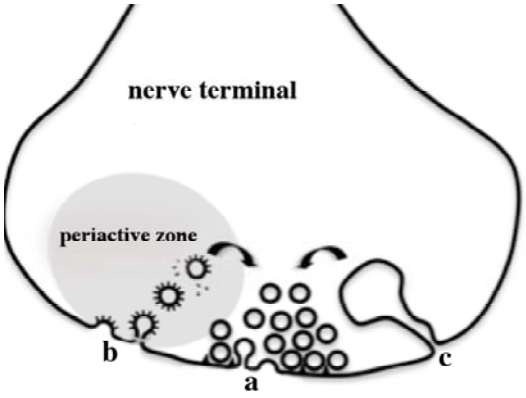

Figure 6. Membrane uptake in the nerve terminal. Synaptic vesicle recycling via kiss n run in the active zone (a), clathrin-mediated- (b) and bulk endocytosis (c) in the periactive zone (grey).

2.1.1 Dynamin and its partners in synaptic vesicle recycling

Synaptic vesicle recycling involves the clathrin coat machinery and the neuronal-specific isoform of dynamin, dynamin 1, which interacts with e.g. endophilin, amphiphysin, syndapin and intersectin, enriched in the nerve terminal[90, 135, 152, 153]. Most endocytic proteins exist in isoforms that are ubiquitously expressed and implied in endocytic events in other cells, or intracellular compartments. Dynamin 2, for instance, is know to participate in intracellular membrane trafficking events with amphiphysin 2[154] and syndapin 2[155]. As mentioned previously, knockdown of dynamin 1 in mice is not embryonically lethal, though it does affect activity-dependent membrane uptake in cultured neurons[90]. It was suggested that dynamin 2 and 3 have overlapping functions and rescue the loss of dynamin 1 in these cells[90]. This suggests a general overlap in the function of distinct isoforms, which may serve to procure crucial endocytic events, such as maintained synaptic transmission.

Endocytic proteins need to localize to the periactive zone to perform their functions during synaptic vesicle recycling. It is been shown that some endocytic

proteins are dephosphorylated by the phosphatase calcineurin in a Ca^{2+}-dependent manner, upon stimulation of the nerve terminal[156]. Amphiphysin, dynamin, epsin, eps15 and synaptojanin are such, so-called dephosphins[156]. Amphiphysin has been shown to re-locate from the synaptic vesicle cluster, to the periactive zone upon stimulation and dephophorylation[157], hence recruitment of endocytic proteins to the periactive zone, is a major mechanism to regulate synaptic vesicle recycling.

Dynamin lacks the ability to interact with the clathrin coat directly and $PI(4,5)P_2$ binding by the PH-domain of dynamin has proven insufficient for proper localization at sites of endocytosis in non-neuronal cells[127]. Also, dynamin PRD deletion mutants fail to localize to endocytic sites, which results in an inhibition of transferrin uptake in these cells[126, 128]. All this suggests that dynamin is dependent on interactions with SH3-domain proteins to localize to the periactive zone in nerve terminals. Several major binding partners of dynamin, enriched in the nerve terminal, are suggested to be required for proper synaptic vesicle recycling.

Recent studies indicate that dynamin may mediate fission in the presence of GTP, independent of any accessory proteins. These fission events require $PI(4,5)P_2$-rich lipid tubes with proper curvature, a sufficient concentration of dynamin and tension of the lipid membrane, which suggests several possible regulatory functions for dynamin binding partners during the fission reaction.

2.2 SYNAPTIC VESICLE RECYCLING AS A MODEL OF MEMBRANE UPTAKE

The nerve terminal provides an excellent model of membrane uptake that may be studies at high spatial and temporal resolution, using EM, live cell imaging and electrophysiological methods. First of all, the organization of the nerve terminal and specifically the area of membrane uptake, is well defined. Synaptic vesicles fuse at the active zone and are re-formed via clathrin-mediated or bulk endocytosis, in separates areas, periactive zones (Fig. 6)[1-3, 79-81]. More over, the exo- endocytosis cycle is a highly regulated, Ca^{2+}-dependent event and may thus be evoked and monitored using various techniques. EM has been a long-standing tool to investigate the morphology of the synapse and has contributed greatly to the understanding of membrane recycling pathways. The role of clathrin in membrane uptake was first suggested from studies of the frog NMJ using EM[158, 159]. Recent developments in cell imaging has provided useful tools to study membrane uptake in nerve terminals live, either by tracking fluorescent markers, such as the lipophilic dye FM1-43 as it incorporates into forming synaptic vesicles, or synaptopHluorin, a GFP-fused vesicle protein, which is quenched when taken up into forming synaptic vesicles. Further more, especially large nerve terminals, such as the mammalian retinal bipolar synapse are routinely used for studies of membrane uptake, using capacitance measurements, which provides analysis of endocytic events at high temporal resolution[151, 160, 161]. Another advantage of studying membrane uptake in nerve terminals is the size of the structures, which allows microinjections of agents to acutely perturb the endocytic process. The squid giant pre-synaptic terminal and synapses in giant reticulospinal axons of the vertebrate lamprey[143, 162-164] are two such model synapses.

3 AIMS

The main goal of my studies was to investigate the roles of dynamin binding SH3-domain containing proteins endophilin, intersectin and syndapin during endocytosis and my specific goals were:

1) To elucidate how the large scaffolding protein intersectin participates in synaptic vesicle recycling and in particular in the recruitment of dynamin to sites of endocytosis.

2) To explore how the membrane remodelling BAR-domain proteins endophilin, and syndapin target dynamin to necks of clathrin-coated pits and participate in the membrane fission reaction.

4 METHODS

4.1 MODEL SYSTEMS

4.1.1 The lamprey giant synapse

We used synapses in giant reticulospinal axons of the vertebrate lamprey to study membrane uptake (**paper I, II, IV**). The advantages of using this synapse as a model of membrane uptake is the high degree of spatial organization compared to most mammalian synapses. Several separate release sites may be found in the same immediate area of the axon and sites of endocytosis are distinguishable from areas of exocytosis (Fig. 7). This allows for ultrastructural studies of the nerve terminal using transmission electron microscopy (EM). Another advantage is, as the name implies, the size of the axons (approximately 100 µm in diameter), which allows microinjections of protein domains, antibodies and other agents to perturb the function of specific proteins, or interactions between binding partners acutely during the endocytic process.

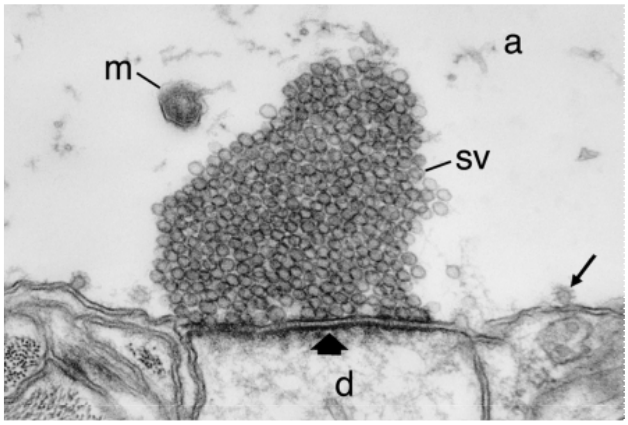

Figure 7. Release site in a giant reticulospinal axon of the vertebrate lamprey stimulated at 5 Hz. Membrane uptake via clathrin- and bulk endocytosis occurs lateral to the active zone (arrow head), in the periactive zones. Note the presence of ccps (arrow). (a) axoplasm, (SV) synaptic vesicle, (m) mitochondria, (d) dendrite.

4.1.2 Cerebral granule neurons and Cos 7 cells

To study membrane uptake is a more dynamic system, we used primary cultures of cerebral granule neurons (CGN) (**paper II**). Membrane uptake was assayed by loading of the styryl dye FM1-43, which incorporates into membrane compartments upon stimulation of the cells and may be visualized using fluorescence microscopy[165], and by re-acidification of synaptopHluorin, a fusion protein of the vesicle protein VAMP2 and a pH-sensitive GFP, which fluoresces when expelled from the acidic environment inside synaptic vesicles upon exocytosis and is quenched (re-acidified) when internalized by endocytosis[166]. We also used cultured Cos 7 cells and assayed clathrin-mediated membrane uptake of fluorescently tagged transferrin, using fluorescence microscopy (**paper III**).

4.2 ELECTRON MICROSCOPY

EM is an excellent tool to visualize cellular processes, such as endocytosis at the subcellular level. It renders a resolution simply not obtainable using optical systems such as live cellular imaging with confocal microscopy or TIRF-M. We have used conventional EM primarily to investigate the morphology of the lamprey nerve terminal (**paper I, II, III, IV**), but also to investigate membrane tubulation properties

of various endocytic proteins, in Cos 7 cells (**paper III**) and on lipid templates *in vitro* (**paper III, IV**). EM requires that tissue samples are fixed, embedded into resin, sectioned and put on grids. We obtained series of ultra-thin sections using diamond knifes and could thus analyze the morphology of lamprey nerve terminals in their entirety. Cryo-EM allows samples to be visualized at high resolution without fixation, as they are plunged into liquid ethane and frozen in solution. We utilized this method to investigate the organization of various endocytic proteins on lipid templates (**paper IV**).

4.3 MICROINJECTIONS

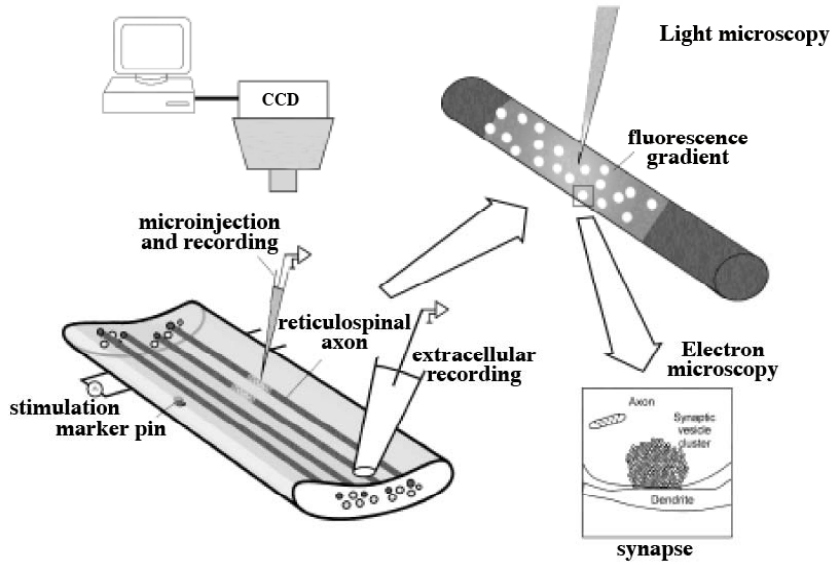

Figure 8. Schematic illustration of the microinjection procedure. Fluorescently tagged agents are injected into lamprey giant reticulospinal lamprey axons. Axons are stimulated, fixed, embedded in resin and sectioned, which allows ultrastructural analysis of the endocytic process in the nerve terminal.

Microinjections into lamprey giant axons allow acute perturbations of the function of various endocytic proteins, or protein-protein interactions occurring during membrane uptake in the living synapse (**paper I, II, III, IV**). Using thin-walled microelectrodes, fluorescently tagged substances are injected into isolated pieces of lamprey spinal cord, kept in solution, at a fixed temperature throughout the procedure. The spread and relative concentration of substances in the axons may be monitored by fluorescence microscopy. Suction electrodes are used to initiate and to monitor activity in the axons.

4.4 PROTEIN LOCALIZATION IN THE NERVE TERMINAL

The subcellular localization of endocytic proteins in nerve terminals was investigated in lamprey reticulospinal axons, mouse hippocampal mossy fibers and Schaffer collaterals and in primary astrocyte cultures, using fluorescence microscopy (**paper I, II**). EM was further used to localize proteins at an ultrastructural level using immunogold labelling (**paper I, II, IV**). This technique allows localization of proteins in the nerve terminal at rest, activity and following perturbations of the endocytic process by microinjections (lamprey). Pre-embedding immunogold labelling to localize endocytic proteins in the lamprey nerve terminal was performed as shown in Figure 9; axons are cut longitudinally after microinjections (upper inset), which exposes endocytic sites to

antibodies, that are applied before the embedding in resin. Post-embedding immunogold labelling allows localization of protein in sections of embedded tissue (lamprey and mouse).

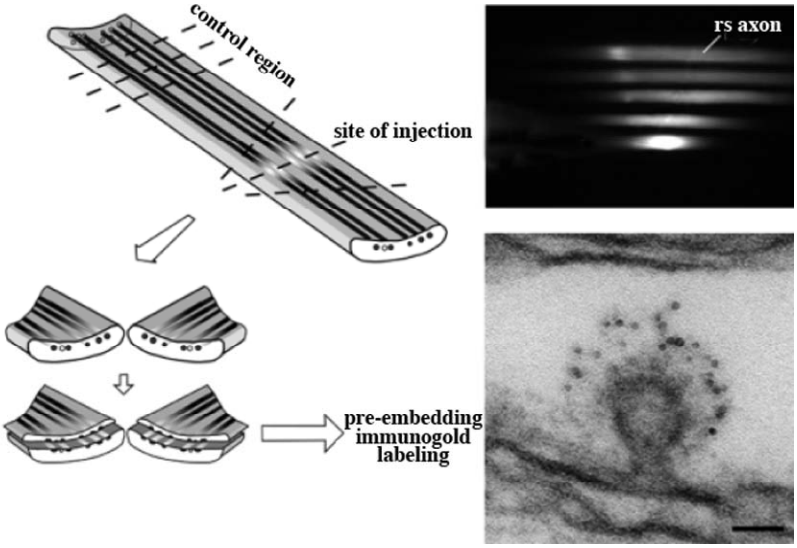

Figure 9. Schematic illustration of the pre-embedding immunogold labelling technique. Isolated pieces of lamprey spinal cord is cut longitudinally, exposing endocytic sites to antibodies for protein localization using EM. Lower inset showing a ccp labelled for dynamin using pre-embedding immunogold labelling.

4.5 ANALYSIS OF PROTEIN-PROTEIN INTERACTIONS

To further investigate the role of specific protein-protein interactions during membrane uptake, we performed various *in vitro* binding assays between full-length endocytic proteins, or various domains of these. Proteins were produced from constructs, generated from lamprey cDNA by cloning (intersectin domains, **paper I, II**), or subcloning into expression vectors, expressed and purified as fusion proteins in *E.coli* using affinity chromatography (**paper I, II, III, IV**), or in insect cells using size exclusion chromatography (**paper IV**). Immuno- and co-immunoprecipitation was performed to assay protein-protein interactions in brain extract (lamprey and rat) (**paper I, II, III, IV**), affinity chromatography was utilized to investigate protein-protein or domain-domain interactions in solution and competition experiments were performed to investigate the effect on various interactions in the presence of blocking agents, such as peptides and antibodies (**paper I, II, III, IV**). To study specific protein-protein interactions in living cells, CGNs and Cos 7 cells were transfected with protein domains and the effect on endocytosis was monitored using fluorescence microscopy (**paper II**).

4.6 CRYSTALLOGRAPHY

The structures of syndapin (full-length and the F-BAR-domain) and the intersectin-AP2 interaction module were solved using X-ray crystallography of protein crystal obtained using the hanging drop vapor diffusion method (**paper III**), or the sitting drop vapor diffusion method (**paper II**).

4.7 ANALYSIS OF PROTEIN-LIPID INTERACTIONS IN *IN VIVO*

The lipid binding property of various endocytic proteins was assayed by sedimentation of lipid-protein samples, followed by SDS-page to analyze the amount of protein

present in the lipid-rich pellet fraction (**paper III, IV**). The lipid bending property was analyzed using EM of the samples, absorbed onto grids and visualized by negative stain, or using cryo-EM (**paper III, IV**). Proteins (full-length, and domains) were also transfected into Cos 7 cells and their *in vivo* membrane tubulation activity was analyzed using fluorescence microscopy and EM (**paper III**).

5 INTERSECTIN AND SCAFFOLDING OF THE ENDOCYTIC PROCESS

5.1 RECRUITMENT OF DYNAMIN TO THE PERIACTIVE ZONE

Mutants of dynamin lacking the PRD fail to localize to sites of endocytosis in non-neuronal cells[126]. This results in a block in clathrin-mediated endocytosis, despite of the ability of dynamin to bind $PI(4,5)P_2$ in the plasma membrane[126]. Therefore, the recruitment of dynamin to the periactive zone in nerve terminals likely depends upon interactions with SH3-domain containing binding proteins[126, 127, 167]. Studies in *Drosophila* and *C.elegans* NMJs show that the homologue of intersectin, Dap160, is required for proper localization of synaptojanin, endophilin, AP180 and dynamin at the endocytic zone[131, 132, 168]. In *Drosophila*, dynamin recruitment to sites of endocytosis is dependent on complex formation between eps15 and intersectin[168]. NMJs in Dap160 null mutants show a depletion of synaptic vesicles and a decrease in FM1-64 dye uptake after stimulation, compared to wt flies[131], which indicates that intersectin is important for synaptic vesicle recycling.

5.1.1 Dynamin and intersectin are re-distributed from the synaptic vesicle cluster upon stimulation and co-localize at ccps in the periactive zone

To elucidate the role of intersectin in the endocytosis process in vertebrate synapses, we investigated its subcellular localization in nerve terminals of lamprey using fluorescence microscopy and EM. Fluorescently tagged antibodies against intersectin were microinjected into lamprey axons and visualized as spots, similar to those seen in axons injected with antibodies against the synaptic vesicle marker synapsin, using fluorescence microscopy (**Paper I**, Fig. 2). Upon stimulation of the lamprey axons to evoke neurotransmission and re-uptake of synaptic membrane, the intersectin labelling was dispersed, which indicates that intersectin re-distributes laterally in the axon upon stimulation (**Paper I**, Fig. 2c vs. d). The ultrastructural localization of intersectin was investigated using EM. Synaptic membrane uptake in these nerve terminals occurs lateral to the active zone, in the periactive zone (**Paper I**, Fig. 3a, white arrow), where ccps are notably absent during rest (**Paper I**, Fig. 3c, f). Under these conditions, both intersectin and dynamin are localized to the synaptic vesicle cluster (**Paper I**, Fig. 3a-c, f). In stimulated nerve terminals on the other hand, ccps are accumulated in the periactive zone and both proteins localize to these structures (Fig 10 and **Paper I**, Fig. 3d, g). Since some recent studies questioned the pre-synaptic localization of intersectin in mammalian synapses[169], we re-examined this using our new antibody, which specifically recognized the A-C SH3-domain region of the mammalian protein. We found that intersectin is present in nerve terminals of mammalian mossy fiber synapses (**Paper II**, Fig. S2). This further strengthens our conclusions based on studies in the lamprey nerve terminal. Thus intersectin and dynamin are present in the synaptic vesicles cluster at rest and at ccps in the periactive zone of stimulated synapses.

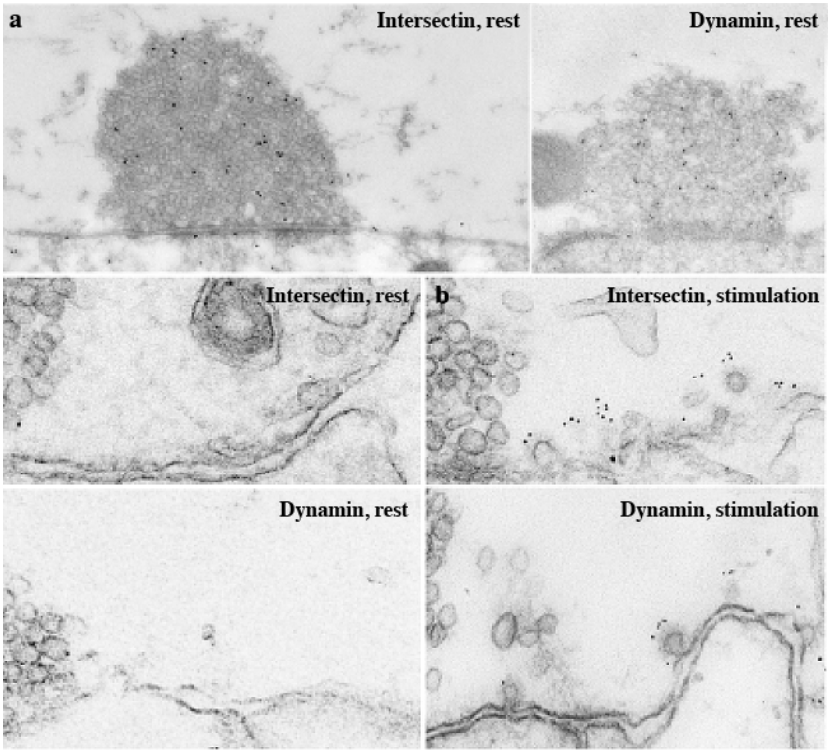

Figure 10. Ultrastructural localization of dynamin and intersectin in the nerve terminal. Dynamin and intersectin co-localize at the synaptic vesicle cluster at rest (a) and at ccps in the periactive zone after stimulation (b).

We also found that phallodin (a marker for filamentous actin) specifically labels the area peripheral to intersectin in lamprey reticulospinal axons (**Paper I**, Fig. 1). Contrary to intersectin, the phalloidin signal remains unchanged after stimulation, which indicates that filamentous actin forms a network surrounding synaptic vesicle release sites in lamprey nerve terminals.

5.1.2 Acute perturbation of intersectin function inhibits clathrin-mediated endocytosis in the lamprey nerve terminal

In addition to dynamin, intersectin interacts with synaptojanin in lamprey and mammalian brain extract, as revealed pull-down and by co-immunoprecipitation (**Paper I**, Fig. 1). Acute perturbation of the intersectin function by microinjections of antibodies, raised against the SH3A-C region (LIS-AC) followed by stimulation of the axons inhibits clathrin-mediated endocytosis in the lamprey giant synapse (**Paper I**, Fig. 5). Clathrin-coated pits, accumulated in the periactive zone are predominately constricted, which is indicative of a block at a late stage of synaptic vesicle recycling, just prior to dynamin-mediated fission (**Paper I**, Figs. 6b and 7b). We also observed large membrane invaginations in the periactive zone, which indicates that intersectin may also participate in early stages of clathrin-mediated endocytosis (**Paper I**, Fig. 5c).

5.1.3 Intersectin regulates the recruitment of dynamin from the synaptic vesicle cluster to the periactive zone

We further investigated if the inhibition of synaptic vesicle recycling was due to a perturbation in the recruitment of dynamin to the periactive zone. Dynamin shares the same pattern of localization as intersectin in the lamprey nerve terminal, the synaptic vesicle cluster at rest and ccps in the periactive zone of stimulated synapses (**Paper I**, Fig. 3b, d). This co-localization suggests that intersectin may recruit dynamin to the periactive zone upon stimulation. Surprisingly, we found that when the function of intersectin was perturbed by the injection of LIS-AC antibodies, dynamin localization to the periactive zone was dramatically increased (Figs 11 and 15d and **Paper I**, Fig. 7).

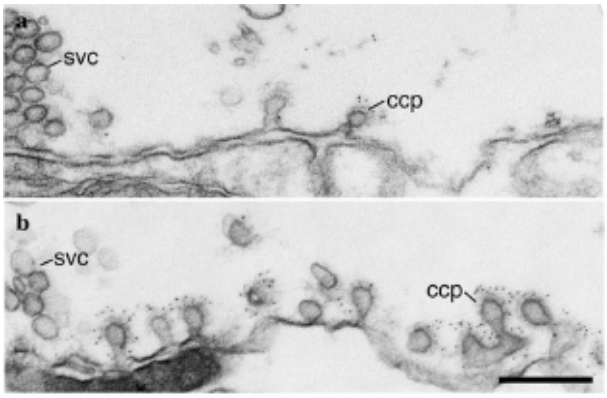

Figure 11. Dynamin localization in the nerve terminal of a lamprey reticulospinal axon stimulated at 5 Hz. Injection of antibodies against intersectin (LIS-AC) causes an increase in dynamin localization in the periactive zone (b), compared to un-injected, stimulated synapses (a).

It was recently shown that *C.elegans* intersectin loss-of-function mutants accumulate more dynamin in their NMJs following stimulation[170]. Taken together, these experiments allow us to suggest that intersectin functions as a negative regulator of dynamin recruitment to the periactive zone.

We propose that intersectin forms a complex with dynamin, which "locks" it in the synaptic vesicles cluster. Upon depolarization of the nerve terminal, Ca^{2+} entry and calcineurin-dependent dephosphorylation of dynamin[157], this complex disassembles, which allows dynamin to be recruited to the periactive zone. This recruitment appears to be a complex regulation of interactions between intersectin and at least one other SH3-domain protein. In vertebrates, amphiphysin resides in the vesicle cluster at rest and has been shown to influence the localization of dynamin at necks of ccps[58, 128]. Amphiphysin is not present in *Drosophila*, which suggests that a number of different proteins may contribute to the recruitment of dynamin.

Another interesting finding is the observation that both dynamin and intersectin are localized at ccps throughout their maturation from shallow to constricted (**Paper I**, Fig. e, f). This is contrary to results obtained using TIRF-M, which suggest that dynamin is recruited to the constricted ccps at the very last stage of endocytosis, just prior to fission of the newly formed vesicle[171]. This observation may well be due to the limited resolution of this method. In these experiments fluorescent spots at sites of membrane budding correspond to GFP-tagged overexpressed proteins and the appearance of these spots is interpreted as *de novo* protein recruitment. An alternate interpretation is that the appearance of spots may be a result of oligomerization of proteins present at the ccps.

In the lamprey nerve terminal, intersectin was localizes almost exclusively at the clathrin coat (**Paper IV**, S1b). Acutely perturbing intersectin function inhibits

endocytosis in the nerve terminal (**Paper I**, Fig, 5), though not as a result of impaired dynamin recruitment to the periactive zone (**Paper I**, Fig. 7).

Thus, recruitment of dynamin to this area is not enough to mediate fission. This suggests that dynamin must be properly targeted to the membrane neck. How intersectin may participate in this process will be discussed in the next section.

5.2 INTERSECTIN CONTROLS PROGRESSION FROM EARLY TO LATE EVENTS IN CLATHRIN-MEDIATED ENDOCYTOSIS

To further investigate the involvement of intersectin at early stages of clathrin-mediated endocytosis, we injected the antibody against the linker region (between SH3A- and B-domain) of lamprey intersectin (LIS-linker) into reticulospinal axons.

5.2.1 Intersectin interacts with the coat machinery by binding to AP2

The linker region of intersectin is not involved in binding to dynamin, which prefers the A, C and E SH3-domains of lamprey intersectin (**Paper I**, Fig. 1d). Interestingly, antibodies against the EH-domain of mammalian intersectin (715), co-immunoprecipitates the clathrin adaptor protein AP2 from brain extract (**Paper II**, Fig. 2a-b). Further investigation show that intersectin binds AP2 via two amino acid peptide sequences (WADF and WDxW), closely resembling those used by other endocytic proteins to interact with the α/β-appendages of AP2 (**Paper II**, Fig. 2e-f)[8, 11, 13, 14]. Point mutations in these two sites impaired intersectin binding to both AP2-appendages (**Paper II**, Fig. 2e-f). Using isothermal titration calorimetry, we found the binding constants for the α- and the β-appendage to be 5 μm, and 490 nm, respectively, indicating that intersectin preferentially interact with the β-appendage of AP (**Paper II**, Fig. 2g). The β-appendage of AP2 is functionally linked to clathrin binding[8]. We then identified the crystal structure of the binding region between intersectin and the α/β-appendages of AP2 (Fig. 12 and **Paper II**, Fig. 3A and B, respectively). The α/β-appendages contain two putative binding sites for accessory proteins, the top site, and the side site[8, 16, 18]. We mutated single residues in each site and investigated the effect on protein binding. Mutation of the top site residues causes a decrease in epsin binding, but does not affect binding of either intersectin (via the WADF) or AP180 (**Paper II**, Fig. 3e). Contrary, mutating the side site results in loss of intersectin and AP180 binding to AP2, while epsin is unaffected. Also, we confirm that the intersectin-AP2 interaction indeed occurs *in vivo* as the proteins co-localize in cultures of primary astrocytes (**Paper II**, Fig. S2f-g).

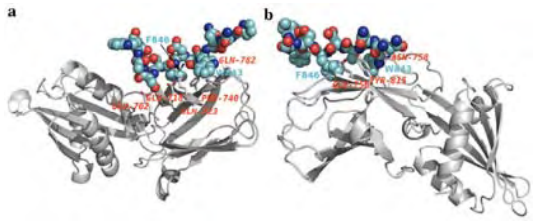

Figure 12. The crystal structure of the intersectin-AP2 interaction module. a) Shows a ribbon diagram of the intersectin-derived peptide (blue) in complex with the AP2 α-appendage (grey). b) Shows a ribbon diagram of the intersectin-derived peptide (blue) in complex with the AP2 β-appendage (grey).

5.2.2 Acute perturbation of the intersectin-AP2 interaction inhibits clathrin-mediated endocytosis at the stage of shallow ccp

To investigate the function of the intersectin-AP2 interaction in vivo, the linker domain was co-expressed with the synaptic vesicle recycling marker synaptopHluorin in CGNs (**Paper II**, Fig. S4a-b). We found that the wt, but not the AP2 binding deficient linker domain of intersectin inhibits re-quenching of synatopHlorin (caused by re-acificiation) in CGNs. This indicates that synaptic vesicle recycling is inhibited if the AP2-intersectin interaction is perturbed. In living lamprey synapses, antibodies against the linker region inhibit synaptic vesicle recycling accompanied by an accumulation of shallow ccps and membrane invaginations in the periactive zone (Fig. 13 and **Paper II**, Fig. 1). This suggests that the involvement of intersectin in early endocytic events is mediated via the interaction between intersectin and the clathrin adaptor AP2.

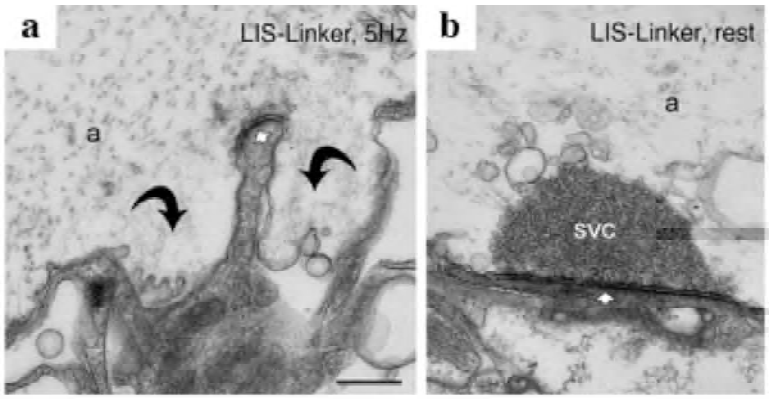

Figure 13. Acute perturbation of the intersectin-AP interaction by microinjection of antibodies against the intersectin linker domain into lamprey giant reticulospinal axons. A) Nerve terminals of injected axons stimulated at 5 Hz. Note the accumulation of ccps in the periactive zone, compared to nerve terminals in nerve terminals kept at rest (b).

5.2.3 The intersectin-AP2 complex regulate intersectin binding to synaptojanin

We further investigated if AP2 binding may influence the role of intersectin during later stages of clathrin-mediated endocytosis. As previously mentioned, intersectin binds a number of endocytic proteins. We tested the binding ability of the intersectin SH3A-C region in the presence of the linker antibody or either of the AP2 appendages and found that under these conditions, intersectin no longer binds the neuronal-specific isoform of synaptojanin (synaptojanin 145), though interestingly dynamin binding was unaffected (**Paper II**, Fig. 4). Synaptojanin antibodies co-immnunoprecipitate intersectin and endophilin from brain extract, though in the presence of the AP2 appendages, the binding to intersectin was lost, while endophilin binding was unaffected. We conclude that intersectin indeed plays an important role during early, as well as late stages of clathrin-mediated endocytosis. This has previously been shown in 3T3 cells, where the SH3A-domain of intersectin blocks transferrin uptake before constriction of ccps, while other SH3-domains cause an arrest later, at the stage of vesicle budding[122]. It is possible that intersectin is a recruitment platform for AP2 at the plasma membrane. Possibly together with eps15, another binding partner of AP2, that has been shown to form a multi-complex with intersectin in *Drosophila* NMJs, which recruits dynamin[168]. The affect on synaptojanin binding is interesting. Synaptojanin is the major protein implicated in uncoating of newly formed vesicles[29, 35, 36]. Intersectin binding to synaptojanin may prevent pre-mature $PI(4,5)P_2$ hydrolysis at the plasma membrane. Release of intersectin from AP2 may be key to unleash synaptojanin and thus initiate uncoating. There are two isoforms of synaptojanin, a long (170 kDa) and a short neuronal-specific isoform (145 kDa). Synaptojanin 170 has been shown to

associate with ccps from early stages of formation, though this isoform is not expressed in neurons and thus does not participate in synaptic vesicle recycling[172]. Endophilin has been shown to recruit both isoforms of synaptojanin to ccps in non-neuronal cells[172]. AP2 binding prevents intersectin from interaction with the neuronal specific isoform of synaptojanin (**Paper II**, Fig. 4). This implies that intersectin may recruit synaptojanin 145 to the coat early to ensure that it is present at the scene for prompt coat disassembly via $PI(4,5)P_2$ hydrolysis when the time comes. Intersectin may precipitate synaptojanin on the coat and later also shuttle it down to the neck in an endophilin-dependent manner, to hydrolyze $PI(4,5)P_2$ and potentially facilitate fission.

5.2.4 Summary

Intersectin interacts with a number of endocytic proteins in the nerve terminal and functions as a master scaffolding protein by regulating the recruitment of the fission GTPase dynamin to the periactive zone and the transition between early, to late stages of clathrin-mediated synaptic vesicle recycling. It binds AP2 and disruption of this interaction allows the recruitment of synaptojanin to ccps.

6 DYNAMIN AND FISSION – INTERPLAY BETWEEN PROTEINS AND THE PLASMA MEMBRANE

6.1 LOCALIZATION OF DYNAMIN AT THE MEMBRANE NECK

The ultimate destination for dynamin in endocytosis is the constricted membrane neck of ccps. As shown in the previous section, intersectin is important to regulate the recruitment of dynamin from the synaptic vesicle cluster, to the periactive zone upon stimulation of the nerve terminal. Acute perturbation of intersectin using LIS-AC antibodies causes a block of synaptic vesicle recycling at the stage of fission, made evident by the accumulation of constricted ccps in the periactive zone (**Paper I**, Fig. 4). Fission is blocked despite of an increase in the amount of dynamin in the periactive zone (**Paper I**, Fig. 7a, c, d). Hence it is not enough to recruit dynamin to the periactvie zone to mediate fission, dynamin must be properly targeted to the neck of the ccp.

6.1.1 Intersectin is required for localization of dynamin at the neck of ccps prior to fission

Fission is a transient event. To study assembly of dynamin on necks of ccps, an event that likely occurs just prior to fission, we microinjected GTPγS, a non-hydrolysable analogue of GTP, which impairs dynamin GTP-hydrolysis. This causes an inhibition of synaptic vesicle recycling in lamprey nerve terminals and a massive accumulation of constricted ccps with elongated necks, decorated by protein spirals (**Paper I**, Fig. 6f and **Paper IV**, Figs. 6 and 3). In the synaptic vesicle recycling process these endocytic intermediates correspond to the stage of clathrin-mediated endocytosis just prior to fission. Similar structures were previously shown in perforated synaptosomes treated with GTPγS[99, 173] and on brain lipid liposomes incubated with cytosol and GTPγS[100]. Amphiphysin, dynamin and synaptojanin were detected on these structures[99, 100, 173]. Not surprisingly, we detected dynamin on elongated necks of endocytic intermediates in synapses injected by GTPγS (Fig. 14a and **Paper IV**, Fig. 3e-f).

To elucidate how dynamin is targeted to these sites, we also investigate the localization of dynamin binding partners at these structures. While dynamin predominately localized to the membrane neck, intersectin localized exclusively to the clathrin coat of these structures (Fig. 14d and **Paper IV**, Fig. S1e).

We microinjected the SH3C-domain of intersectin into lamprey synapses, to acutely and specifically perturb the interaction between dynamin and intersectin. This resulted in an inhibition of clathrin-mediated endocytosis at the stage of fission, similar to what was observed after injections of the LIS-AC antibody (**Paper I**, Fig. 4a-b). Interestingly, while the antibody caused an increase in dynamin recruitment to the periactive zone, the SH3C-domain had the opposite effect (Fig. 15b and **Paper I**, Fig. 7b-d). Dynamin does not properly localize to necks of ccps in synapses injected with the intersectin SH3C-domain and more over, the protein spiral observed on constricted necks of ccps is evidently thinner compared to those seen on ccps in control (un-injected, stimulated) synapses (**Paper I**, Fig. 6c-f). This is in agreement with results from NMJs of Dap160 *null* mutant flies, where ccps with thin spirals accumulated following stimulation[131]. Amphiphysin still localized to ccps in injected synapses, despite the loss of dynamin (**Paper I**, Fig. 7d). This indicates that intersectin does not

regulate the localization of amphiphysin to necks of ccps and further that amphiphysin cannot "rescue" dynamin localization at necks when the dynamin-intersectin interaction is impaired. This suggests a role of intersectin up-stream of amphiphysin during regulation of fission.

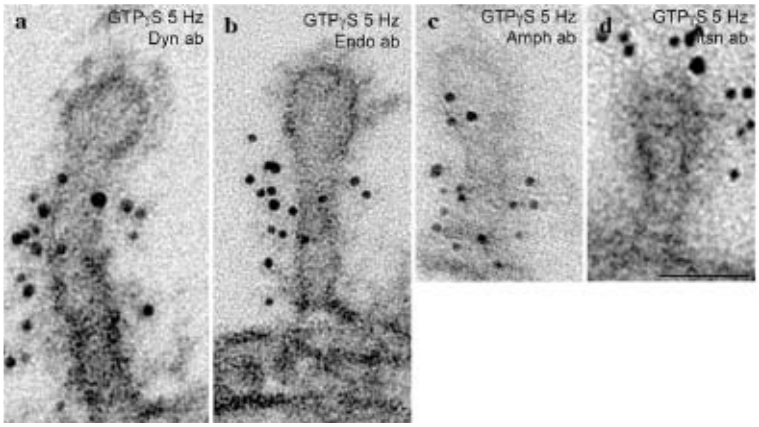

Figure 14. Localization of dynamin, endophilin, amphiphysin and intersectin on endocytic intermediates accumulated in nerve terminals of lamprey reticulospinal axons injected with GTPγS and stimulated at 5 Hz. Dynamin (a) and amphiphysin (c) label the entire neck, while endophilin localizes specifically to the coat-neck interface (b) and intersectin exclusively to the clathin coat (d).

6.1.2 Endophilin is a template for dynamin at the membrane neck

The BAR-domain containing proteins amphiphysin and endophilin were also detected on endocytic intermediates accumulated in nerve terminals of axons injected with GTPγS (Fig. 14b and c and **Paper IV**, Figs. 3 and S1). Interestingly, while intersectin specifically localized to the clathrin coat and amphiphysin was evenly distributed along the entire structure, endophilin had a distinct localization at the base of the clathrin coat (Fig. 14 and **Paper IV**, Fig. 3b-c). Endophilin was further detected on ccps at all stages of maturation, but almost exclusively at the rim of the coat (**Paper IV**, Figs. 1b and S1a-c). We also confirmed that endophilin resides in the synaptic vesicle cluster during rest (**Paper IV**, Fig. S1a) and in the periactive zone, specifically associated with ccps and synaptic vesicles, following stimulation (**Paper IV**, Fig. 1a). This suggests that endophilin also re-locates from the cluster to the periactive zone upon stimulation, just like intersectin, amphiphysin and dynamin. Amphiphysin contains a CLAP domain and has been suggested to be an integral part of the clathrin coat in vertebrate synapses[174]. Endophilin lacks binding ability to the coat machinery and may be recruited to sites of endocytosis via its BAR-domain, or in a complex with a binding partner, such as oligophrenin[175]. Recent studies show that in cells from conditional knockout mice of dynamin 1 and 2, endophilin promotes formation of constricted ccps with elongated necks[176]. Endophilin co-localizes with actin in these cells and it was further shown that blocking actin polymerization by latrunculin B causes a dispersion of endophilin labelling and prevents formation of long, narrow necks. These results suggest that actin is required to regulate the recruitment of endophilin to the membrane.

To investigate the role of endophilin in dynamin targeting to necks of ccps, we microinjected PP19, a peptide corresponding to a sequence in the synaptojanin PRD, which binds endophilin in brain extract[36, 135] and perturbs the interaction between dynamin and endophilin in vitro (**Paper IV**, Fig. S2a-b). PP19 was previously shown to cause an inhibition of synaptic vesicle recycling in lamprey nerve terminals, attributed to impaired synaptojanin-mediated uncoating (**Paper IV**, Figs. 2a, b and S2c)[36]. In this study, we show that PP19 indeed causes an accumulation of clathrin-coated vesicles (ccvs), but also a profound increase in the number of ccps accumulated in periactive zones of injected synapses. This indicated that PP19 inhibits fission, as

well as uncoating (Fig 15 and **Paper IV**, Figs. 2 and S2c). Moreover, dynamin localization at necks of ccps was significantly decreased in synapses injected with PP19 (Fig 15b, d and **Paper IV**, Fig. 2). This indicates that the interaction between endophilin and dynamin is required for proper targeting of dynamin to necks of ccps during synaptic vesicle recycling.

Thus, dynamin recruitment to the periactive zone and proper localization at necks of ccps requires interactions with intersectin and endophilin (**Paper I**, Figs. 4 and 7 and **Paper IV**, Fig. 2). Intersectin is localized at the clathrin coat of ccps (**Paper I**, Fig. 1 and **Paper IV** 4, Fig. S1b). We propose that it functions as a protein shuttler, directing proteins, such as dynamin and synaptojanin from the coat, down to the neck. Intersectin regulates the amount of dynamin that is recruited to the periactvie zone, but another protein is involved in the actual recruitment, possible amphiphysin, or SNX9. Endophilin has a distinct distribution on constricted necks (**Paper IV**, Fig. 3). It localizes at the coat-neck interface. We suggest that endophilin is a template for dynamin at the necks of ccps and that it promotes targeting of dynamin to these sites after it has been recruited to the periactive zone.

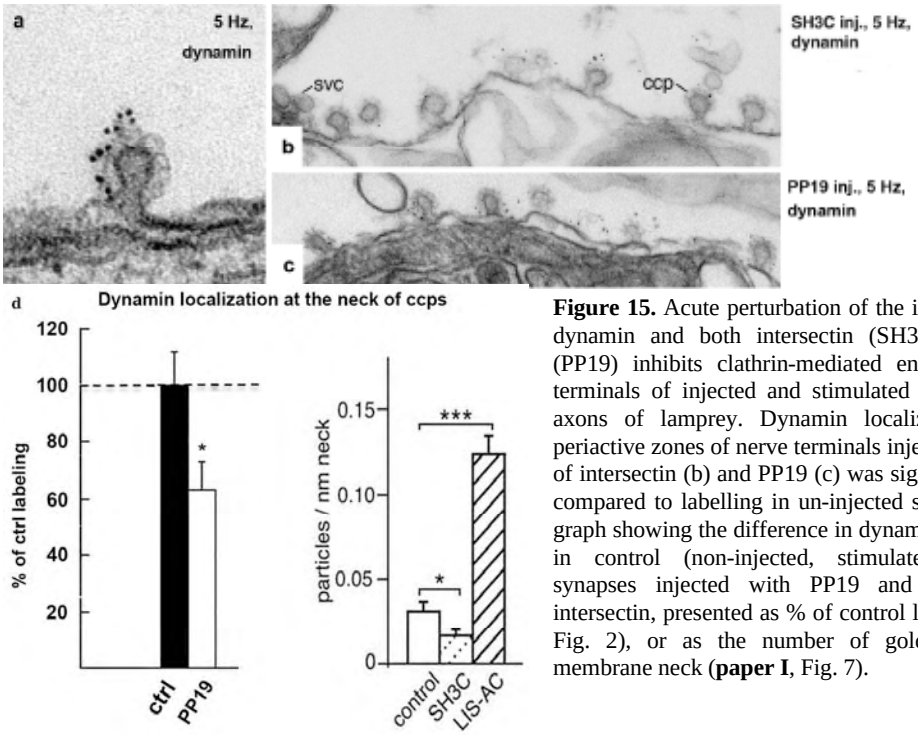

Figure 15. Acute perturbation of the interactions between dynamin and both intersectin (SH3C) and endophilin (PP19) inhibits clathrin-mediated endocytosis in nerve terminals of injected and stimulated giant reticulospinal axons of lamprey. Dynamin localization on ccps in periactive zones of nerve terminals injected with the SH3C of intersectin (b) and PP19 (c) was significantly decreased compared to labelling in un-injected synapses (a). d) Bar graph showing the difference in dynamin labelling on ccps in control (non-injected, stimulated synapses) and synapses injected with PP19 and SH3C-domain of intersectin, presented as % of control labelling (**paper IV**, Fig. 2), or as the number of gold particles/nm ccp membrane neck (**paper I**, Fig. 7).

6.2 MEMBRANE FISSION – A ONE MAN SHOW?

Recent *in vitro* studies show that dynamin-mediated fission is influenced by the concentration of dynamin and the curvature and tension of the membrane[68, 118]. This implies involvement of accessory proteins to facilitate the fission reaction. We have shown that endophilin is important to target dynamin to necks of ccps in living synapses (**Paper IV**, Fig. 2). Endophiln has the ability to deform liposomes into lipid tubes *in vitro* [57], corresponding to the diameter of necks of ccps observed in nerve terminals *in vivo* (**Paper IV**, Fig. 4). We further show that endophilin has a very strategic localization on ccps, at the base of the coat (Fig 14 and **Paper IV**, Fig. 3b-c),

ideal to concentrate dynamin at this site. We wanted to further investigate the role of endophilin in fission.

6.2.1 Endophilin and dynamin form a distinct complex in vitro and on necks of constricted ccp in living synapses

Endophilin and dynamin both localize to the neck of ccps in periactive zones of synapses injection with GTPγS (Fig 14 and **Paper IV**, Fig. 3). It was previously shown that dynamin co-assembles with both endophilin and amphiphysin on tubulated lipsomes in vitro [52, 58]. We used an *in vitro* assay to further investigate the interactions between dynamin and endophilin on membrane templates. We found that the proteins indeed co-assemble into spirals when added together to liposomes (Fig 16a, b, d and **Paper IV**, Fig. 4) and that these protein spirals are distinct from those formed by dynamin alone under the same conditions (Fig 16c and **Paper IV**, Fig. 4d, i vs. f, j). The pitch of the endophilin-dynamin spiral was wider and the diameter of the endophilin-dynamin decorated lipid tubes was significantly thinner (**Paper IV**, Fig. 4). Over all, these structures resembled constricted necks of ccps decorated by protein spirals in lamprey nerve terminals injected with GTPγS (Fig 16d *vs.* e and **Paper IV**, Fig. 4d vs. e).

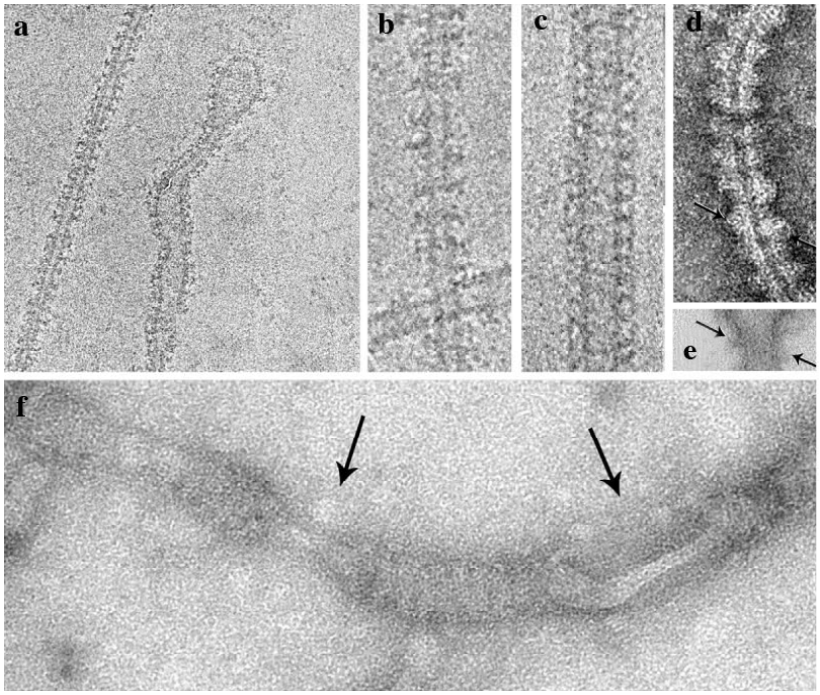

Figure 16. Endophilin-dynamin complex formation. (a, b, d) endophilin, and dynamin form a complex on lipid templates *in vitro* , that resembles spirals decorating necks of ccps in living synapses (e) and is distinct from complexes formed by dynamin alone (c). Addition of PP19 causes disassembly of the endophilin-dynamin complex formed in lipid templates *in vitro* (f).

Interestingly, the diameter of the endophilin-dynamin decorated lipid tube corresponded to that of dynamin-decorated tubes in the presence of GTP, which has supposedly undergone constriction upon GTP binding[104, 119, 120]. Also, we observed that the average inner lumen diameter of these tubes was very small, in range of what is proposed to result in hemi-fission, and subsequent fission[177]. This suggests that endophilin-dynamin complex formation promotes a constriction of the membrane in the absence of GTP, which may be important for subsequent events in fission.

We also investigated the ability of dynamin to bind- and tubulate liposomes in vitro and found both of these properties to be significantly increased by the presence of endophilin (**Paper IV**, Fig. 5), further supporting a role of endophilin as a template for dynamin at necks of ccps.

Endophilin-dynamin spirals assembled on liposomes *in vitro* failed to form in the presence of PP19 (Fig. 16d and **Paper IV**, Fig. 5). This indicates that PP19 inhibits fission in lamprey nerve terminals by preventing the formation of an endophilin-dynamin complex on necks of ccps (**Paper IV**, Fig. 2). This further suggests that endophilin-dynamin complex formation is required for fission. To test this notion, we injected GTPγS into lamprey synapses in the presence of PP19, to investigate how perturbation of the endophilin-dynamin interaction affected the formation of protein spirals on necks of ccps. We found a massive accumulation of ccps in periactive zones of these synapses. Interestingly, all ccps had short necks and lacked obvious decoration by protein spirals, seen in synapses injected with GTPγS alone (**Paper IV**, Fig. 6).

We propose that endophilin and dynamin form a pre-fission complex on necks of ccps and that this complex is required for fission as it promotes further recruitment of dynamin and constricts the membrane neck, which may lead to hemi-fission and then GTP-mediated fission.

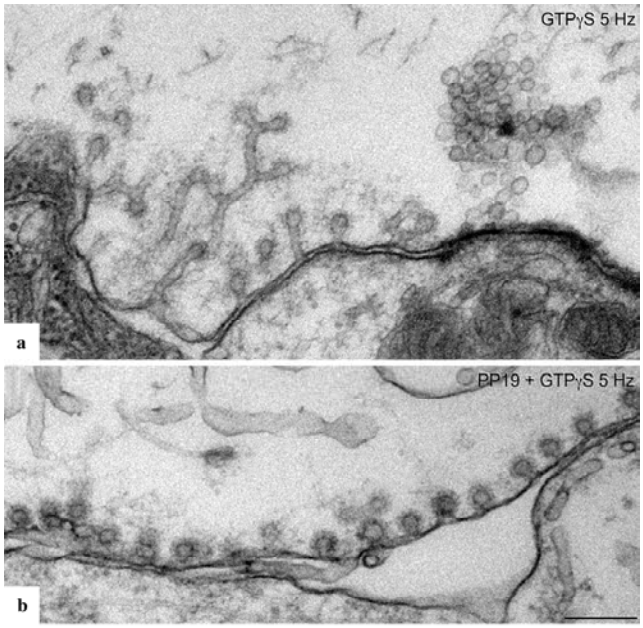

Figure 17. Acute perturbation of endophilin-dynamin interactions inhibits formation of protein spirals on necks of ccps in stimulated nerve terminals of lamprey giant reticulospinal axons. GTPγS causes a massive accumulation of ccps in periactive zones of injected and stimulated axons (a). The presence of PP19 in synapses, prevents this effect of GTPγS (b).

6.2.2 Summary

Dynamin localization at necks of ccps is crucial for assembly of a protein spiral and for subsequent fission of newly formed clathrin-coated vesicles. Intersectin and endophilin promote targeting of dynamin to necks of ccps in lamprey nerve terminals and perturbation of the interaction between either of these proteins and dynamin inhibits the fission reaction. Further, both intersectin and endophilin localize to endocytic intermediates trapped at the stage of fission by the presence of GTPγS. The distinct distributions of both proteins, intersectin on the coat and endophilin at the coat-neck interface, suggest they influence dynamin localization at distinct steps in the endocytic process. Endophilin assembles into a complex with dynamin on lipid templates *in vitro*, resembling protein spirals formed *in vivo*. Endophilin also promotes recruitment of dynamin to lipid templates and the formation of endophilin-dynamin decorated lipid tube *in vitro*. Blocking the interaction between endophilin and dynamin in living synapses prevents efficient fission and formation of elongated necks, decorated by proteins spirals.

6.3 THE DYNAMIN–BAR PROTEIN INTERACTION – RECIPROCAL REGULATION?

Syndapin is another dynamin-binding BAR-domain protein enriched in the nerve terminal[153]. The syndapin-dynamin interaction is required for synaptic vesicle recycling in cultured neurons[144, 178, 179]. However, syndapin does not localize to ccps in periactive zones of stimulation lamprey nerve terminals and does not seem to be involved in clathrin-mediated endocytosis to recycle synaptic vesicles[143, 144]. Though it has been shown to function in activity-dependent bulk synaptic membrane retrieval[143, 144]. It was suggested that syndapin is recruited to large membrane invaginations via its ability to sense (larger) curvature[51]. Syndapin may in turn promote targeting of dynamin to these sites, which subsequently leads to fission of large membrane invaginations during bulk-retrieval in the nerve terminal.

6.3.1 Intra-molecular regulation of the BAR membrane tubulation properties

It was previously suggested that full-length syndapin may be regulated in its tubulation activity by intra-molecular interactions[180]. We found that full-length syndapin has a limited ability to promote formation of membrane tubes, compared to the isolated F-BAR-domain, when expressed in cells (**Paper III**, Fig. 1). To understand the basis of this suggested auto-inhibition, we obtained and analyzed the crystal structure of the syndapin (full-length, and the F-BAR-domain) (Fig 18 and **Paper III**, Figs. 2 and S2). We identified a wedge loop in the F-BAR-domain dimer, which makes syndapin unique in the BAR-domain family (**Paper III**, Fig. 2). This wedge loop and the positive charge of the BAR-domain dimer are necessary to promote formation of membrane tubes *in vivo* (**Paper III**, Fig. 1d-f, j). The crystal structure of the syndapin F-BAR-domain further suggested an intra-molecular interaction between the F-BAR- and the SH3-domain of syndapin (**Paper III**, Fig. 2). *In vitro* binding assays using recombinant domains of syndapin, further show that the SH3-domain indeed binds the F-BAR-domain (**Paper III**, Fig. S3). This interaction proved to be functionally important, as an SH3-domain mutant of syndapin, unable to bind the F-BAR-domain, exhibited tubulation activity close to the isolated F-BAR-domain when expressed in cells (**Paper III**, Fig. g, j).

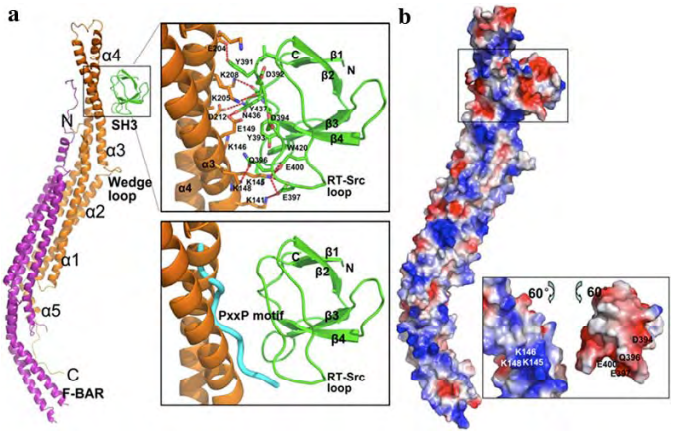

Figure 18. The crystal structure of full-length syndapin. a) Shows a possible association between the SH3-domain (green) and the F-BAR-domain full-

6.3.2 Dynamin regulates the auto-inhibition of syndapin

We wanted to test what may regulate this auto-inhibition of syndapin. Syndapin binds dynamin and N-WASP and may function as a link between dynamin-mediated fission and the actin cytoskeleton during endcoytosis[76, 140, 145]. Full-length syndapin was co-expressed with dynamin K44A (a dynamin mutant that cannot hydrolyze GTP[181]) in Cos 7 cells. This co-expression caused an increase in tubulation in vivo (**Paper III**, Figs. S6 and S7). To further investigate the role of dynamin in the regulation of syndapin lipid binding and tubulation, recombinant syndapin was added to liposomes. Lipid binding was assessed by sedimentation of the protein-lipid sample using ultra-centrifugation and the amount of protein in the pellet was considered "lipid-bound". The limited lipid tubulation ability of full-length syndapin observed *in viv*o was confirmed using EM analysis of protein-lipid samples visualized by negative stain. Incubating lipsomes with the F-BAR-domain, the syndapin SH3-mutant unable to bind the F-BAR-domain, or full-length syndapin in the presence of a peptide corresponding to the syndapin binding sequence of the dynamin PRD, significantly increased the amount of tubes observed *in vitro*, compared to full-length syndapin alone (Fig 19 and **Paper III**, Fig. 5).

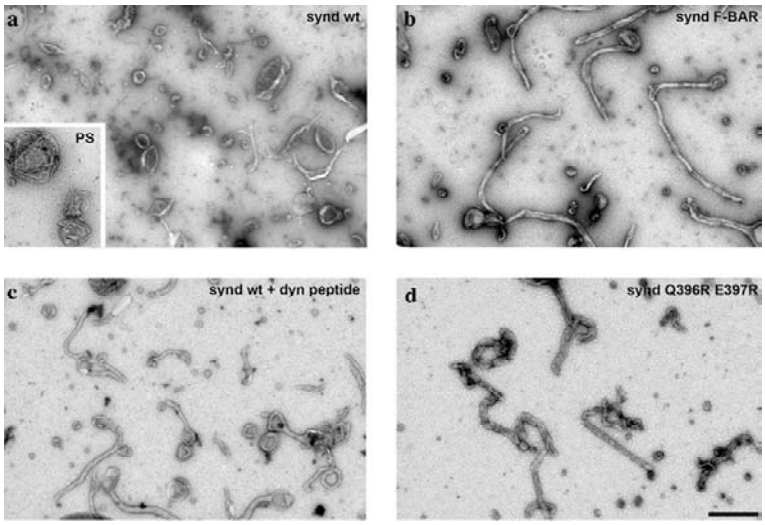

Figure 19. Tubulation activity of full-length syndapin is significantly increased by perturbation of the intra-molecular SH3-F-BAR interaction is perturbed. a) Syndapin full-length has limited ability to tubulate PS liposomes (inset), compared to the isolated F-BAR-domain (b). Addition of a syndapin SH3-domain binding peptide of dynamin promotes liposome tubulation (c). Mutating the SH3-domain of full-length syndapin, rendering it unable to bind the F-BAR also promotes its ability to tubulate PS liposome *in vitro* (d).

Interestingly, full-length syndapin was able to bind liposomes and we observed no increase in lipid binding corresponding to the increase in tubulation, promoted by either the F-BAR-domain, the dynamin peptide, or the SH3-mutant (**Paper III**, Fig. S5). This indicates that syndapin lipid sensing and bending are two distinct properties, as suggested for other BAR-domain proteins and that the tubulation is regulated by intra-molecular interactions between the F-BAR- and the SH3-domain.

We wanted to investigate if this auto-inhibition is a general trait among BAR-domain proteins to regulate their membrane modulating property. ΔSH3-Endophilin was shown to induce tubules in HUVEC cells, while wt did not[56]. We analyzed the binding of the SH3-domains of endophilin, and amphiphysin, to their respective BAR-domains and identified possible intra-molecular interactions for both proteins *in vitro* (**Paper III**, Fig. 4e-f). We show that the auto-inhibition of syndapin does not regulate membrane binding, merely the tubulation activity. This suggests that BAR-domain proteins may function as membrane templates for dynamin and at the same time, be regulated by dynamin in their ability to bend the membrane. We suggest a functional

dynamin-syndapin partnership in bulk-membrane retrieval, where syndapin, functions as a membrane template for dynamin, just like endophilin, while dynamin acts to “unlock” syndapin, mediating membrane tubulating. In light of this finding, a potential reciprocal regulation of dynamin-mediated fission and BAR-mediated membrane remodelling, it is of great interest to explore if this holds true for other BAR-proteins, such as endophilin.

6.3.3 Summary

Intra-molecular interaction between the SH3- and the F-BAR-domain of syndapin auto-inhibits efficient membrane tubulation *in vivo* and *in vitro*. Co-expression of dynamin rescues this ability in cells and *in vitro*, the presence of a dynamin peptide, promotes syndapin membrane tubulation.

7 A NEW MODEL FOR DYNAMIN-MEDIATED FISSION

Intersectin is a master scaffolder of the clathrin-mediated endocytosis process, regulating recruitment of early and late effectors to the periactive zone and the forming clathrin-coated pit. Intersectin is also a coat-to-neck shuttler at ccps, sitting on the clathrin coat directing dynamin and synaptojanin to the neck, where they interact with endophilin. Endophilin and syndapin are lipid membrane-scaffolding templates for dynamin at the membrane. Dynamin and its BAR-domain protein partners form functional complexes, which spatially and temporally coordinate membrane deformation and dynamin GTPase activity, to mediate fission.

During clathrin-mediated endocytosis, endophilin promotes recruitment of dynamin to necks of ccps, where they form a distinct complex. This pre-fission complex coordinates dynamin localization and assembly, with BAR-domain protein mediated membrane tubulation to constrict the membrane neck. The pre-fission complex promotes dynamin-mediated fission by 1) remodelling the membrane – clustering $PI(4,5)P_2$ and creating curvature of the neck, 2) organizing further dynamin assembly – resulting in the formation of a dynamin spiral anchored to the complex, 3) shielding the neck from synaptojanin – causing a phase separation along the length of the ccp. Phase separation of lipid tubes of a certain curvature is sufficient to mediate fission[68, 117]. GTP-binding and hydrolysis induced conformational change in the pre-fission complex and the dynamin-spiral further facilitates the scission reaction and causes disassembly of the protein complex, allowing synaptojanin to mediate hydrolysis of $PI(4,5)P_2$.

During bulk endocytosis, the syndapin-dynamin interaction is important[144]. We propose that these proteins also assemble into a pre-fission complex to mediate fission of larger membrane chunks during synaptic vesicle recycling.

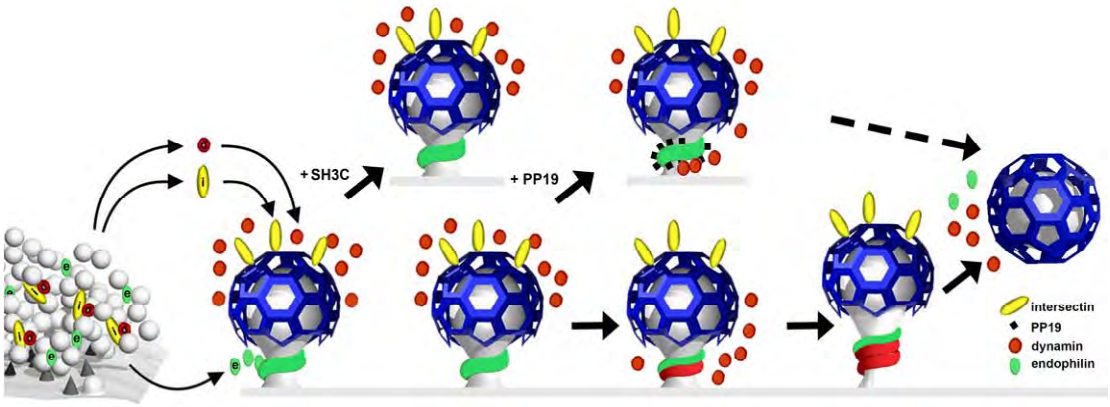

Figure 20. Schematic illustration of the events leading up to plasma membrane fission. Intersectin, endophilin, and dynamin are localized to the synaptic vesicle cluster at rest. Upon stimulation, intersectin regulates the recruitment of dynamin to the periactive zone. Intersectin shuttles dynamin and synaptojanin from the coat to the neck. Endophilin functions as a membrane neck template for dynamin and together they assemble into a pre-fission complex that mediates constriction of the membrane neck, further recruitment of dynamin and neck protection from synaptojanin.

8 CONCLUSIONS

Intersectin and endophilin are localized in the vesicle cluster in synapses at rest and control proper localization of dynamin at ccps in the periactive zone during synaptic vesicle recycling.

Intersectin interacts with the clathrin adaptor complex AP2 at early stages of clathin-mediated endocytosis in synapses. Inhibition of this interaction by clathrin allows the intersectin SH3A-domain to bind the inositol phosphatase synaptojanin, which promotes uncoating of clathrin-coated vesicles.

Intersectin and endophilin have distinct localizations on coated pits during synaptic vesicle recycling. Intersectin is localized strictly to the coat region, while endophilin is accumulated at the rim of the coat and at the neck.

Endophilin promotes recruitment of dynamin to the neck of coated pits in living synapses and the proteins form a complex at the neck of coated pits.

Syndapin membrane tubulation activity is regulated by the intra-molecular interaction between its F-BAR and SH3-domains. Dynamin binding to the SH3-domain prevents this interaction and promotes the syndapin membrane tubulation activity both *in vivo* and *in vitro*.

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