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Coordination of endocytosis at the synaptic periactive zone

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Stockholm 2006

Cover: A montage of a confocal image of a lamprey reticulospinal synapse labeled with amphiphysin antibodies (green) and phalloidin (red), and an electron micrograph of a synapse labeled using post-embedding technique with amphiphysin (5 nm gold particles) and actin (10 m gold particles) antibodies.

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ISBN 91-7140-683-2
Larserics Digital Print AB 2006

Abstract

Neurons in the central nervous system communicate via specialized junctions called synapses. Neurotransmitter-filled vesicles are clustered at these junctions, where they are released in response to synaptic activity. To sustain reliable neurotransmission the synaptic vesicles are recycled locally. At least two recycling mechanisms, bulk and clathrin-mediated endocytosis, occur in a region adjacent the active zone, the periactive zone. Clathrin-mediated endocytosis is dependent on a number of protein-protein and protein-lipid interactions that drive clathrin polymerization, membrane invagination, and scission.

The aim of this thesis is to investigate how compensatory endocytosis is coordinated in the synaptic periactive zone. The studies are focused on actin dynamics in the periactive zone, molecular adaptations of the endocytic machinery in synapses with different firing properties, recruitment of endocytic proteins to the periactive zone, and the molecular steps leading to fission of clathrin-coated intermediates.

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

- Synaptic activity promotes actin filament formation in the periactive zone and dispersion of synapsin from the synaptic vesicle cluster. Perturbation of synapsin function leads to a disruption of actin filament formation.
- Endocytic proteins dynamin, amphiphysin, and intersectin redistribute from the synaptic vesicle cluster to the periactive zone in response to synaptic activity.
- Synapses established by axons with a tonic activity pattern have higher levels of synapsin, dynamin, amphiphysin, and intersectin compared to those with phasic activity. This may contribute to the resistance to fatigue displayed by tonic synapses.
- Dynamin, amphiphysin, and intersectin are recruited to initial stages during clathrin-coated vesicle formation.
- Disruption of amphiphysin's interaction with coat components by antibodies results in formation of clathrin-coated pits with an abnormal shape. This suggests that amphiphysin is an integral component of the clathrin coat.
- Dynamin is recruited to clathrin-coated pits independently of intersectin. However, intersectin is important for the recruitment of dynamin to the fission complex.

Taken together these results show that clathrin-mediated membrane retrieval in synapses require coordinated trafficking of endocytic proteins between active and periactive zones.

Keywords: synapse, endocytosis, clathrin, dynamin, amphiphysin, synapsin, intersectin, actin cytoskeleton, immunogold electron microscopy.

List of publications

This thesis is based on the following original papers that are reprinted in the second part of the thesis. They will be referred to in the text by their roman numerals:

Paper I: Bloom O., **Evergren E.**, Tomilin N., Kjaerulff O., Löw P., Brodin L., Pieribone VA., Greengard P., Shupliakov O. (2003) Colocalization of synapsin and actin during synaptic vesicle recycling. J Cell Biol 161:737-747.

Paper II: **Evergren E.**, Tomilin N., Vasylieva E., Sergeeva V., Bloom O., Gad H., Capani F., Shupliakov O. (2004) A pre-embedding immunogold approach for detection of synaptic endocytic proteins in situ. J Neurosci Methods 135:169-174.

Paper III: **Evergren E.**, Marcucci M., Tomilin N., Löw P., Slepnev V., Andersson F., Gad H., Brodin L., De Camilli P., Shupliakov O. (2004). Amphiphysin is a component of clathrin coats formed during synaptic vesicle recycling at the lamprey giant synapse. Traffic 5, 514-528.

Paper IV: **Evergren E.**, Gad H., Walther K., Sundborger A., Jiao W., Eriksson Y., Koh T.-W., Masich S., Tomilin N., Bellen H. J., Shupliakov O. Perturbation of intersectin/Dap160 severly affects synaptic membrane uptake during endocytosis. Manuscript.

Paper V: Evergren E., Zotova E., Brodin L., Shupliakov O. Differential efficiency of the endocytic machinery in tonic and phasic synapses. Submitted.

Abbreviations:

AP180	adaptor protein 180 kD
Arp2/3	actin related proteins 2/3
BAR	Bin/Amphiphsyin/Rvs
Cdc42	cell division cycle 42
CaM	calmodulin
DH	Dbl homology
EH	Eps 15 homolgy
Eps15	epidermal growth factor receptor pathway substrate 15
GTPγS	guanosine 5-O-(3-thiotriphosphate)
N-WASP	neuronal Wiskott-Alrich Syndrom protein
PH	pleckstrim homology
PIP_2	phosphatidylinositol bisphosphate
PKA	protein kinase A
SH3	Src homology 3
SNAP-25	soluble NSF attachment protein 25 kD
SNX9	sorting nexin 9

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Introduction

Communication between neurons in the central nervous system is a fundamental biological process, which takes place at specialized junctions called synapses. The synapse is a highly organized structure with a cluster of neurotransmitter-filled vesicles tethered to a specialized region of the plasma membrane called the active zone. As an action potential reaches the synapse and depolarizes the axonal membrane, voltage-gated Ca^{2+}-channels are opened. Channel opening promotes Ca^{2+} influx, which in turn leads to fusion of synaptic vesicles at the active zone and release of neurotransmitters. The neurotransmitter diffuses across the synaptic cleft to the neighboring neuron where it binds to receptors and triggers an electrical signal.

To reliably sustain neurotransmission and preserve the general organization of synapses, the synaptic vesicles have to be recycled rapidly and locally. Several mechanisms for replenishment have been demonstrated in synapses: fast endocytosis, bulk endocytosis, and clathrin-mediated endocytosis (Kavalali, 2006). At least two of them, bulk and clathrin-mediated endocytosis, occur in the region adjacent to the active zone, referred to as the periactive zone. The periactive zone is a region surrounding active zones where endocytic intermediates are formed during neurotransmission (Gad et al., 1998; Teng et al., 1999; Teng and Wilkinson, 2000). In fruitfly neuromuscular junctions (NMJs) an enrichment of endocytic proteins was observed in this region, i.e., dynamin, intersectin, and adaptor protein complex 2 (AP2) (Chang and Balice-Gordon, 2000; Estes et al., 1996; Gonzalez-Gaitan and Jäckle, 1997; Roos and Kelly, 1998; Roos and Kelly, 1999). In vertebrate synapses, the periactive zone is in addition delimited by an actin-rich cytomatrix (Bloom et al., 2003; Sankaranarayanan et al., 2003; Shupliakov et al., 2002), which proliferates during synaptic activity (Bloom et al., 2003; Shupliakov et al., 2002). The dynamic properties of the actin cytoskeleton suggest that it is an important functional component of the synapse. However, the role of the actin cytoskeleton in the synaptic vesicle cycle still remains obscure (Dillon and Goda, 2005).

Clathrin-mediated endocytosis is a major pathway for recycling of synaptic vesicles and preserving the general organization of synapses during neurotransmitter release. A network of protein-protein interactions and protein-lipid interactions drives the polymerization of clathrin to an invaginated coated intermediate, and then to a constricted clathrin-coated pit that is severed from the membrane and finally uncoated, resulting in a newly formed synaptic vesicle. In addition to clathrin two other components are crucial for this process: AP2, which interacts with clathrin and the integral synaptic vesicle membrane protein synaptotagmin on the plasma membrane, and the GTPase dynamin, which is a key component of the fission complex. Over the recent years the importance of accessory proteins have been highlighted in a number of studies (Murthy and De Camilli, 2003; Slepnev and De Camilli, 2000). Clathrin coat formation is a sequential process that is initiated by formation of a clathrin lattice, which is connected to the plasma membrane via AP2 and AP180 (Ford et al., 2001). Several endocytic accessory proteins have been implicated in membrane bending

and clathrin polymerization, e.g., amphiphysin, endophilin, and epsin (Murthy and De Camilli, 2003). Further, in addition to the GTPase dynamin; amphiphysin, intersectin, and endophilin have been shown to promote scission of clathrin-coated vesicles (Gad et al., 2000; Ringstad et al., 1999; Shupliakov et al., 1997; Simpson et al., 1999; Takei et al., 1999). In this thesis the function of three endocytic proteins; dynamin, amphiphysin, and intersectin is further investigated.

Dynamin is a large GTPase that has the ability to oligomerize and form spirals around lipid tubules (Hinshaw, 2000). The N-terminal contains a GTPase domain, followed by a PH domain that mediates interactions with phosphoinositides. The C terminal part contains the GTPase effector domain (GED) that can interact with itself and activate the GTPase domain, and a proline-rich domain (PRD) that mediates interactions with SH3 domain-containing proteins, i.e., amphiphysin, intersectin, endophilin, syndapin, cortactin, and profilin (Praefcke and McMahon, 2004). It is not known how dynamin mediates fission. Three models have been proposed: (1) the dynamin spiral constricts upon GTP hydrolysis causing severing of the membrane (Chen et al., 2004; Danino et al., 2004; Sweitzer and Hinshaw, 1998; Zhang and Hinshaw, 2001); (2) the GTPase activity causes a lengthwise conformational change of the dynamin spiral leading to membrane fission (Stowell et al., 1999); and (3) the dynamin spiral acts as a molecular switch, which recruits other molecules to perform the scission process (Sever et al., 2000). At the light microscopic level dynamin has been shown to accumulate at endocytic sites during synaptic activity in *Drosophila* NMJs (Estes et al., 1996). However, its localization in relation to different stages of coated intermediates is unclear. Dynamin has been localized to the plasma membrane in non-neuronal cells during endocytosis (Damke et al., 1994; Iversen et al., 2003). In synapses, however, the involvement of dynamin at early stages remains unclear (Takei et al., 1995a). It also remains elusive how dynamin is associated with the clathrin-coat, however, a number of experiments strongly suggest that an interaction with an SH3 domain-containing protein is required (Shpetner et al., 1996; Shupliakov et al., 1997; Wigge et al., 1997b).

The major interaction partner of dynamin in mammalian brain extract is **amphiphysin**. It has been proposed to recruit dynamin to clathrin-coated pits via its C-terminal SH3 domain (Shupliakov et al., 1997; Wigge et al., 1997b). Amphiphysins have a N-terminal BAR domain that associates with and bends lipid membranes (Peter et al., 2004; Takei et al., 1999), and mediates dimerization (Peter et al., 2004; Wigge et al., 1997a). It also interacts with the clathrin coat through its clathrin and AP2 binding (CLAP) domain (Ramjaun and McPherson, 1998; Slepnev et al., 2000). These interactions promote clathrin polymerization (Farsad et al., 2003; Miele et al., 2004). *In vitro* experiments have demonstrated that co-assembly of dynamin and amphiphysin on lipid tubules enhances the membrane severing capacity of dynamin (Takei et al., 1999). Surprisingly, amphiphysin I knockout mice are viable but display slightly impaired synaptic vesicle recycling (Di Paolo et al., 2002). Moreover, *Drosophila* NMJs lack amphiphysin (Leventis et al., 2001; Razzaq et al., 2001; Zelhof et al., 2001). Thus, the role of amphiphysin has to be further investigated to elucidate whether its function can be taken over by other SH3 domain-containing proteins in vertebrate synapses.

Another interaction partner of dynamin, which can bind via three SH3 domains, is **intersectin.** It is a large scaffolding protein that has been proposed to be an important recruiting module during membrane trafficking events (Koh et al., 2004; Marie et al., 2004; Roos and Kelly, 1998). The long isoform of intersectin is comprised of two EH domains and a coiled-coil domain, which binds epsin, Eps15, and SNAP-25 (Okamoto et al., 1999; Sengar et al., 1999), five SH3 domains that interact with dynamin,

synapsin, synaptojanin, and N-WASP (Hussain et al., 2001; Okamoto et al., 1999; Zamanian et al., 2003), and a PH domain that interacts with phospholipids. Its C terminus contains a DH domain, which functions as a guanine exchange factor for Cdc42 (Zamanian et al., 2003), indicating a role for intersectin in the organization of the actin cytoskeleton (Hussain et al., 2001; McPherson, 2002). *Drosophila* mutants have severe defects in synaptic membrane recycling (Koh et al., 2004; Marie et al., 2004), which was accompanied by reduced protein levels of dynamin, synaptojanin, endophilin, and synapsin. It is localized to the periactive zone in both active and inactive *Drosophila* NMJs, suggesting a role in organization of proteins at periactive zones (Roos and Kelly, 1998; Roos and Kelly, 1999). However, neither the functional role of intersectin in a central synapse nor its localization at high resolution have been addressed.

To study the coordination of proteins involved in synaptic vesicle recycling at the periactive zone I used the **giant reticulospinal synapse** of lamprey as a model system (Shupliakov and Brodin, 2000). It is an *en passant* glutamatergic synapse located in the spinal cord that is important in locomotor activity. The giant reticulospinal axon has a diameter of 80-100 μm, is unmyelinated, unbranched, and runs along the length of the spinal cord. These features allow for microinjection of reagents that can perturb specific protein-protein interactions in the presynaptic compartment, which will spread through diffusion up to several hundred μm from the injection site. Ultrastructural analysis of the effects produced by a reagent is performed at different distances from the injection site to evaluate the effect of different concentrations. Furthermore, the reticulospinal synapse is well suited for subcellular localization of synaptic proteins due to its size and unique morphology (the synaptic vesicle cluster in this synapse is surrounded by a large volume of axoplasmic matrix). In small mammalian hippocampal terminals where the vesicle pool is enclosed in a confined membrane compartment, it is more difficult to distinguish the vesicle cluster from vesicles in transit from the periactive zone. A pre-embedding immunogold technique was developed to obtain a strong signal and a high resolution for labeling of proteins in the periactive zone (Evergren et al., 2004). The size of the axon allows them to be readily opened, which permits gold-conjugated antibodies to easily diffuse through the axoplasm and label endocytic intermediates in three dimensions without using detergents, which are required to disrupt cellular membranes in conventional immunogold procedures. We combined this approach with the microinjection technique to monitor the localization of dynamin after acute perturbation of its interaction to intersectin (Evergren et al., manuscript). Thus, the lamprey reticulospinal synapse provides unique possibilities to acutely perturb protein function and simultaneously localize interaction partners with a high resolution.

This thesis will focus on how compensatory endocytosis is coordinated in the synaptic periactive zone, and it is divided into three main sections. The first section studies the role of actin dynamics in cluster organization and periactive zone function. The second section addresses molecular adaptations of the endocytic machinery in two types of glutamate synapse with different activities. The third section will discuss implications of recruitment of endocytic proteins to the periactive zone, and the molecular steps leading to fission of clathrin-coated vesicles from the plasma membrane.

Actin dynamics in synaptic transmission

Actin filaments (F-actin) are composed of monomers (G-actin) assembled into a two-stranded helix. G-actin monomers are held together by weak noncovalent interactions in polymers, which allow F-actin to rapidly assemble and disassemble. This is an important feature for creating a dynamic cytoskeleton. Actin polymerization is faster at the barbed end compared to the pointed end. This creates a net elongation in the barbed end of actin filaments, which is called actin treadmilling. Actin treadmilling requires energy, which is provided by ATP hydrolysis. Actin filament assembly involves a number of actin regulating proteins *in vivo* (Dillon and Goda, 2005; Janmey and Lindberg, 2004). The importance of actin dynamics in the presynaptic compartment has been difficult to establish due to the requirement for actin in cell survival and the seemingly contradictory results obtained in different types of synapse. Nevertheless actin has been proposed to be involved in several steps of the synaptic vesicle cycle: regulating synaptic vesicle clustering, mobilization, fusion, and recycling (Dillon and Goda, 2005).

Actin in synaptic vesicle cluster organization

Chemical synapses in the central nervous system share common morphological characteristics. They contain synaptic vesicles, which are associated in a tight cluster at the active zone. Synaptic vesicles are proposed to be held together by a filamentous matrix composed of synapsin and actin (Hirokawa et al., 1989; Landis et al., 1988). The size of the synaptic vesicle cluster varies between different types of synapse from a couple hundred vesicles in small hippocampal boutons to tens of thousands of vesicles in giant synapses, i.e., Calyx of Held or lamprey reticulospinal synapse (Gustafsson et al., 2002; Satzler et al., 2002). These differences might be due to variations in activity patterns, release probability, and efficiency of recycling. Today we have a limited understanding of how these structural differences affect neurotransmission functionally.

The most prevalent view on the organization of the synaptic vesicle cluster suggests that vesicles are held together by a meshwork of actin filaments and by the vesicle associated protein synapsin I (Hirokawa et al., 1989; Landis et al., 1988). Synapsin I binds to phospholipids in the vesicle membrane and can simultaneously interact with actin filaments via two high affinity interactions (Greengard et al., 1994). These interactions are regulated by phosphorylation by CaM kinase I/II and PKA, which reduces the affinity for phospholipids and F-actin. Dephosphorylation of synapsin I has been demonstrated to facilitate neurotransmitter release in the squid giant synapse (Llinas et al., 1991; Llinas et al., 1985). These observations led to the hypothesis that synapsin facilitates tethering of vesicles in the cluster. Indeed, synapsin knockout mice or acute perturbation in lamprey reticulospinal synapses result in dispersion of synaptic vesicles in terminals (Fig. 1C; paper I; Gitler et al., 2004; Li et al., 1995; Pieribone et al., 1995; Takei et al., 1995b), leading to enhanced synaptic depression (Gitler et al., 2004; Li et al., 1995; Pieribone et al., 1995; Rosahl et al., 1995). Synapsin I is localized to the synaptic vesicle cluster in resting synapses (paper I; Pieribone et al., 1995). In stimulated

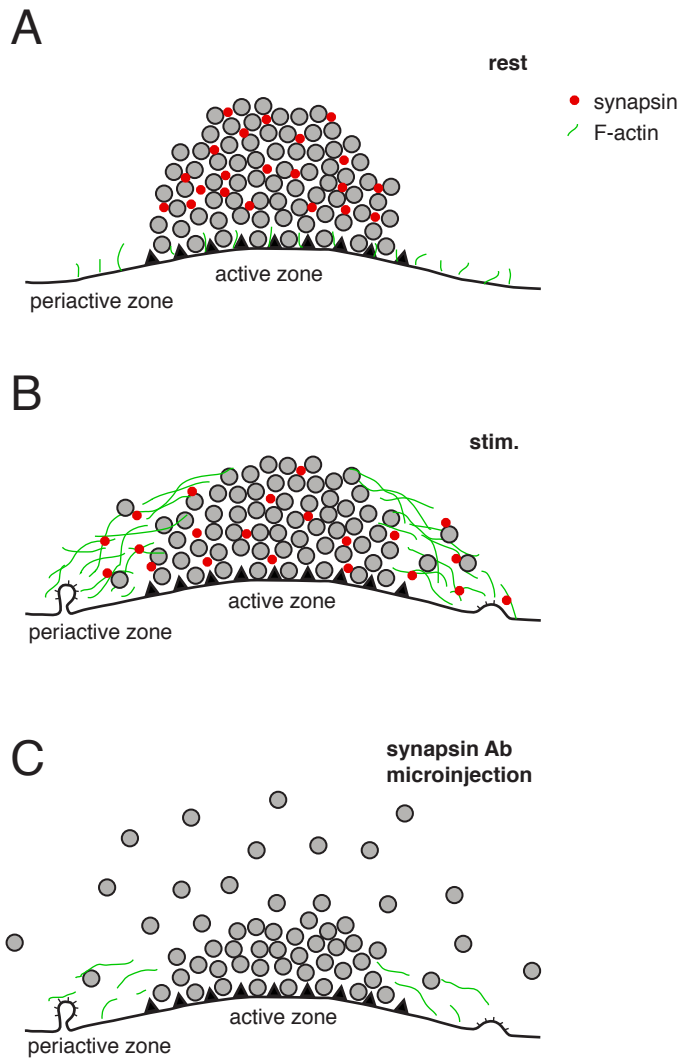

Figure 1: A dynamic actin cytoskeleton proliferates in the synaptic periactive zone upon synaptic activity.

(A) Filamentous actin is not prominent in the synaptic vesicle cluster or the periactive zone in a resting synapse. Some F-actin is present in the active zone and the periactive zone. The actin-nucleating protein synapsin is localized to the distal pool of the vesicle cluster in resting terminals. (B) In a synapse stimulated at a physiological rate an actin-rich cytomatrix is formed in the periactive zone. Within the actin matrix uncoated synaptic vesicles are associated with filaments. Clathrin-coated intermediates are also associated with actin filaments. Synapsin is evenly distributed within the synaptic vesicle cluster and is in addition present in the actin cytomatrix in the periactive zone. (C) Microinjection of synapsin antibodies, raised against the E-domain of synapsin, in reticulospinal synapses followed by stimulation leads to a dispersion of the distal pool of the vesicle cluster and a reduction of the actin filaments in the periactive zone together with a perturbation of transport of vesicles within the periactive zone, which results in migration of vesicles into the axoplasm outside synaptic regions.

synaptic terminals synapsin I dissociates from the vesicle cluster (Fig. 1A-B; paper I; Chi et al., 2001) and diffuses to the periactive zone where it associates with cytoskeletal filaments (probably F-actin) and synaptic vesicles (paper I, II). The dispersion of synapsins makes mobilization of vesicles in the distal reserve pool possible (Chi et al., 2001). Synapsin I is important for synaptic vesicle clustering, which is independent of its actin nucleating activity (Benfenati et al., 1989) and F-actin bundling properties demonstrated *in vitro* (Bähler and Greengard, 1987). This implicates that these properties are not important in the cluster but rather in the periactive zone.

Synapsin might not be solely responsible for controlling the clustering of the reserve pool, but other protein-protein interactions might also play an important role. Recent data from central synapses have shown that the endocytic proteins dynamin, amphiphysin, intersectin, and synaptojanin are localized to the synaptic vesicle cluster in addition to endocytic intermediates (Fig. 2A; paper III, IV, V; Haffner et al., 1997). This suggests that they may participate in organization of synaptic vesicles at release sites too. These proteins are scaffolding proteins that interact with each other and with phosphoinositides. They may form a stabilizing protein matrix that holds the vesicles together at the active zone. Thus, the vast number of intermolecular interactions observed for endocytic proteins in *in vitro* assays (Slepnev and De Camilli, 2000) might not be exclusively important during synaptic vesicle membrane reuptake but also during compartmentalization of proteins in the synaptic vesicle cluster. Supporting evidence for this model comes from acute perturbations of interactions between intersectin, dynamin, and synapsin where vesicles in the middle of the cluster were no longer held together as tightly as in control synapses (paper IV). In summary, several proteins involved in synaptic vesicle recycling have been localized to the vesicle pool where they may facilitate clustering of synaptic vesicles.

Monitoring of the dynamic localization of actin in living hippocampal or reticulospinal neurons demonstrates that the majority of F-actin is not localized to the synaptic vesicle cluster (Sankaranarayanan et al., 2003; Shupliakov et al., 2002). There is a small pool of F-actin in the active zone (Fig. 1A; paper I; Morales et al., 2000; Phillips et al., 2001), which probably needs to be depolymerized in order to permit neurotransmitter release (Morales et al., 2000). However, the biggest share of the actin detected in the synaptic vesicle cluster is in monomeric form (Morales et al., 2000; Sankaranarayanan et al., 2003; Zhang and Benson, 2002). Thus, filamentous actin is not a major structural component of the synaptic vesicle cluster, but rather has a function at the synaptic periactive zone during synaptic activity (paper I). Supporting this notion, exposure of synapses at rest to toxins that promote actin depolymerization did not disrupt clustering of synaptic vesicles (Sankaranarayanan et al., 2003; Shupliakov et al., 2002; Zhang and Benson, 2002). Thus, F-actin is predominantly a structural component of the periactive zone rather than of the distal pool of the synaptic vesicle cluster.

Actin in periactive zone organization

Polymerization of actin within the distal synaptic vesicle pool does not seem to occur in mature synapses. Thus, synapsin's actin nucleating and polymerizing properties do not seem to be required for organizing vesicles in a tight cluster. Indeed, monitoring of the dynamic localization of actin in living hippocampal or reticulospinal neurons demonstrates that a large pool of actin is localized to the synaptic periactive zone (Sankaranarayanan et al., 2003; Shupliakov et al., 2002). This pool is predominantly composed of F-actin (Fig. 1A-B; paper I; Shupliakov et al., 2002). The functional relevance of these findings was addressed in paper I. The phosphorylation-controlled dispersion of synapsin I from the vesicle pool observed at light microscopic level in hippocampal neurons (Chi et al.,

2001) did not define which subcellular compartments synapsin cycles between during synaptic activity. Immunolocalization of synapsin at the ultrastructural level in lamprey demonstrated that it is associated with the actin cytomatrix and the vesicles therein but not with endocytic intermediates (Fig. 1B; paper I; II). Furthermore, injections of antibodies against synapsin leads to a reduction of actin cytomatrix in the periactive zone, and a dispersion of vesicles that normally are confined to the matrix (Fig. 1C; paper I). Thus, the well-established synapsin-actin interaction described biochemically may be more relevant to processes in the periactive zone than in the vesicle clusters. Synapsin's ability to simultaneously bind synaptic vesicles and actin may be functionally important in the periactive zone for trafficking of uncoated synaptic vesicles to the synaptic vesicle cluster. Several studies have suggested that F-actin is involved in trafficking of synaptic vesicles and an abnormal migration of vesicles after perturbation of actin has been reported (Sakaba and Neher, 2003; Shupliakov et al., 2002). This function for actin has also been proposed for trafficking of endocytosed vesicles in fibroblasts (Merrifield et al., 2002). Disruption of the actin cytoskeleton in small hippocampal neurons, however, did not affect synaptic vesicle trafficking (Sankaranarayanan et al., 2003). It is possible that the migration of vesicles in small central synaptic terminals, which have a mechanical boundary created by the plasma membrane, is less dependent on actin cytoskeleton dynamics. Thus, the actin cytomatrix in the periactive zone might be functionally more important in large central synapses, e.g., Calyx of Held, mossy fiber terminals in cerebellum, and lamprey reticulospinal synapse, where the vesicles may disperse significantly after F-actin disassembly. The rate of return of synapsin to the synaptic vesicle cluster is slower than its dispersion rate, but similar to the reclustering rate of vesicles (Chi et al., 2001; Li and Murthy, 2001). This suggests that dephosphorylated synapsin reassociates with newly formed vesicles in the periactive zone (paper I, II) and is transported back with vesicles to the cluster.

Apart from the synapsins, several other proteins have been reported to link the endocytic machinery to the actin cytoskeleton in non-neuronal cells: cortactin, N-WASP, intersectin, profilin, syndapin, and amphiphysin (Schafer, 2004). Thus, regulation of the actin cytoskeleton is a complex process with many accessory proteins involved. The functional connections between the actin cytoskeleton and the endocytic machinery are not well understood, however, it has been demonstrated that depolymerization of the cortical actin cytoskeleton is required for efficient endocytosis (Trifaro and Vitale, 1993).

Amphiphysin, dynamin, and intersectin are colocalized with actin in the periactive zone of action potential stimulated synapses (Fig. 2B; paper III; IV). Amphiphysin has been implicated in regulation of actin dynamics in neurite growth cones (Mundigl et al., 1998). However, the molecular link between amphiphysin and actin in vertebrate synapses remains to be demonstrated.

Intersectin interacts with several important proteins regulating actin polymerization (Hussain et al., 2001; Roos and Kelly, 1998). It may facilitate the formation of actin filaments by recruiting the Arp2/3 regulating protein N-WASP and functioning as a guanine exchange factor for Cdc42 (Hussain et al., 2001; Roos and Kelly, 1998). Recent studies have shown that the major binding partner of intersectin, dynamin, also interacts with proteins that function in actin regulation, such as cortactin and syndapin (Merrifield et al., 2005; Orth and McNiven, 2003). It is possible that intersectin and dynamin form a complex with actin-regulating proteins, which controls different steps of actin filament formation. The exact role of intersectin in the regulation of the cytoskeleton at synaptic sites requires further studies. In synapses microinjected with intersectin antibodies we observe a reduction of

the actin matrix at the periactive zone around membrane invaginations, which may be related to the proposed function of intersectin in the organization of actin cytoskeleton. Depolymerization of cortical actin leads to membrane ruffling (Itoh et al., 2005; Qualmann and Kelly, 2000), which may explain the occurrence of highly convoluted membrane invaginations after perturbation of intersectin function (paper IV). In contrast, reducing the filamentous actin in synapses by introducing C2 botulinum toxin or disrupting PIP_2 levels in the periactive zone was not accompanied by membrane ruffles directed towards the axonal cylinder in the periactive zone (Morgan et al., 2004; Shupliakov et al., 2002), thus suggesting that other proteins may be involved (Itoh et al., 2005). Thus, the role of a dynamic actin cytoskeleton in the periactive zone has many interesting aspects, which require further investigation.

Actin could potentially also be involved in mechanisms regulating the change in mode of endocytosis. Three endocytic pathways have been shown to operate in parallel (Royle and Lagnado, 2003). The change in endocytosis rate at different stimulation frequencies might be a reflection of a switch between fast, clathrin-mediated, and bulk endocytosis. The different pathways are likely to use different sets of endocytic proteins that might share the same members, e.g. dynamin. When the intersectin-dynamin interaction is disrupted *in vivo* large membrane folds appear (paper IV), which are similar to bulk membrane intermediates (Takei et al., 1996). Therefore, changing the efficiency of recruitment of one or several endocytic proteins might shift the balance of endocytosis to another pathway.

Coordination of endocytic proteins during the synaptic vesicle cycle

Another function of the actin cytoskeleton in the periactive zone could be to control the redistribution of synaptic proteins participating in membrane recycling (Fig. 2). Localization of dynamin, amphiphysin, intersectin, and synapsin at ultrastructural level demonstrated that in resting synapses endocytic proteins are primarily accumulated in the synaptic vesicle cluster and not the periactive zone (Fig. 1A, 2A; paper I-V). Dynamin has been reported to have a cytosolic localization based on biochemical fractionation analysis of synaptosomes and light microscopic localization (Estes et al., 1996; Hinshaw, 2000). Our results demonstrate that it is compartmentalized to a large extent in the synaptic cluster in vertebrate synapses (paper IV). A high local concentration of endocytic proteins in the synaptic vesicle cluster might be a critical mechanism for efficient protein recruitment to sites of endocytosis. Then how is the redistribution of proteins from the synaptic vesicle cluster to the periactive zone mediated?

Synapsin I dissociates from synaptic vesicles in stimulated hippocampal neurons and disperses from the vesicle cluster (Benfenati et al., 1989; Chi et al., 2001). The fact that dispersion of synapsin from the synaptic vesicle cluster is faster than dispersion of synaptic vesicle membrane demonstrates that it is not redistributed to the periactive zone via the plasma membrane (Fig. 2C; Chi et al., 2001). Dynamin, amphiphysin, and intersectin are recruited to early stages of endocytic intermediates in response to neurotransmission (paper II-V). This requires dissociation from the vesicle cluster upon action potential stimulation. Dissociation could be governed by dephosphorylation, which has been demonstrated to regulate interactions between multiple proteins involved in synaptic vesicle trafficking *in vitro* (Slepnev et al., 1998). However, for proteins such as amphiphysin and intersectin, containing a lipid-binding domain, recruitment via the vesicle membrane is also feasible (Fig. 2C). For example, intersectin is present in both the cytosolic and membrane fraction of non-neuronal cells (Predescu et al., 2003). Small amounts of amphiphysin, dynamin, and intersectin are detected on non-clathrin coated plasma mem-

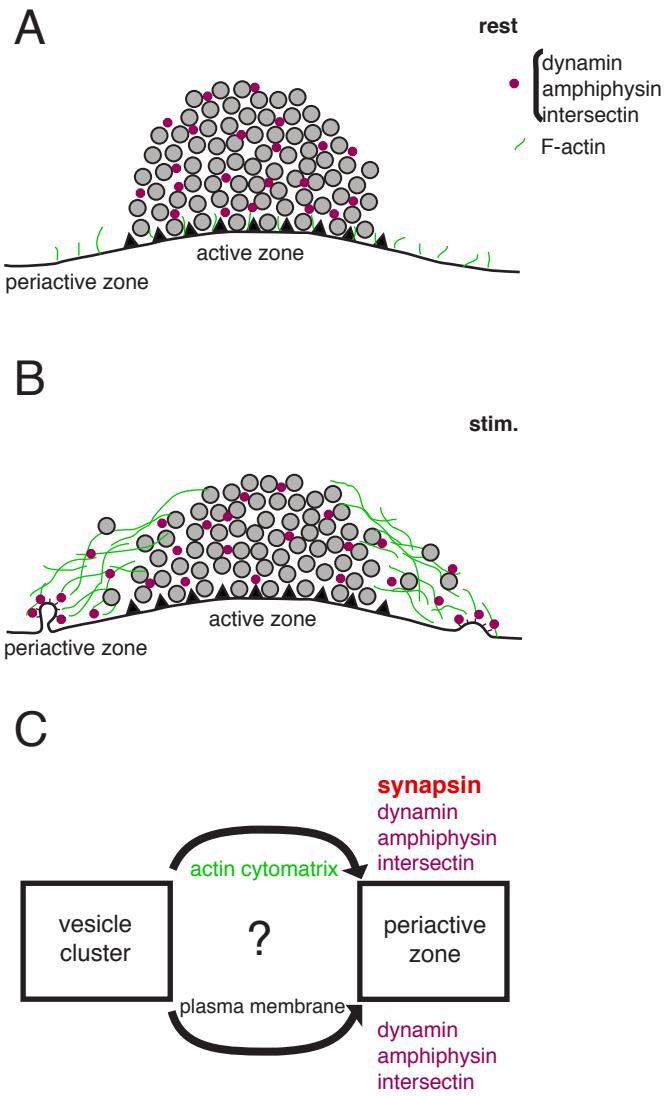

Figure 2: Endocytic proteins are compartmentalized in the resting synaptic vesicle cluster and are redistributed to the periactive zone upon stimulation.
(A) The endocytic proteins dynamin, amphiphysin, and intersectin are accumulated in the synaptic vesicle cluster at rest. (B) In an active synapse these proteins are recruited to the periactive zone, where endocytosis occurs. They are found both on clathrin-coated intermediates and in the actin-rich cytomatrix. (C) Schematic representation of recruitment pathways for synapsin, amphiphysin, dynamin and intersectin during synaptic activity. The endocytic proteins could be recruited to the periactive zone either via the synaptic vesicle membrane or through the actin cytomatrix. The route of transport may differ for proteins with different interaction domains.

brane regions in the periactive zone (paper III; IV). Furthermore, amphiphysin, dynamin, and intersectin are found in the actin cytomatrix at the periactive zone (paper III; IV) where they could be in transit to the periactive zone, and/or returning to the vesicle cluster (Fig. 2B). A redistribution of amphiphysin in the cluster toward the rim also favours a transit route through the actin cytomatrix (Fig. 2C; paper III). Regulation of actin dynamics could thus be a mechanism for limiting dispersion of endocytic proteins to the periactive zone and thereby regulating the efficiency of endocytosis. Synapsin, intersectin, N-WASP, and syndapin are all proteins shown to be involved in synaptic vesicle trafficking and regulation of actin dynamics. The increasing evidence for a connection between dynamin and the actin cytoskeleton could be important in several steps of the synaptic vesicle cycle (Andersson et al., 2003; Merrifield et al., 2005; Schafer, 2004; Yarar et al., 2005).

Adaptation of the synaptic endocytic machinery

Synapses in the central and peripheral nervous system show diverse functional properties most likely as a consequence of adaptation to the specific requirements at a given synapse. One type of synaptic adaptation, which involves the presynaptic compartment, is the specialization to tonic and phasic synapses (Atwood and Karunanithi, 2002). In synapses adapted to tonic activity, transmitter release can be sustained at high levels for extended periods of time. In phasic synapses, on the other hand, transmitter release normally occurs intermittently and when tonic stimulation is applied a fatigue of transmitter release is seen. This difference appears to be due to ATP supply, neurotransmitter synthesis, organization of release sites and Ca^{2+} channels at the active zone, and mode of transmitter release (Atwood and Karunanithi, 2002; Brodin et al., 1999; Harata et al., 2006; Zhai and Bellen, 2004). In light of these findings it is plausible that the molecular machinery that controls recycling of synaptic vesicles is adapted according to the rate of release of tonic and phasic synapses. We investigated this by comparing levels of endocytic proteins in two types of glutamatergic synapses in the lamprey spinal cord with tonic and phasic activity respectively. Our results demonstrate that the level of dynamin, amphiphysin, intersectin, and synapsin labeling is significantly higher in tonic synapses compared to phasic ones (paper V). Furthermore, a prolonged depolarization of a tonic synapse does not result in depletion of the synaptic vesicle pool, which is not the case for the phasic synapse exposed to the same conditions. We suggest that this is due to a higher capacity of the recycling machinery in these synapses that keeps up with the rate of fusion when vesicle collapse occurs (paper V). Thus, our results indicate that an increased efficiency of the endocytic machinery in a synapse may be one of the adaptations underlying the ability to sustain neurotransmission at high rates.

Endocytic proteins at clathrin-coated intermediates

Recruitment of endocytic proteins to clathrin-coated intermediates

Dynamin is required at the final step of clathrin-coated vesicle formation, mediating fission of the constricted neck (Hinshaw, 2000). Our experiments show that in synapses dynamin is recruited to clathrin-coated pits already from early stages (papers II, IV). This finding is in agreement with earlier observations of Damke et al. (Damke et al., 1994) in non-neuronal cells. Since dynamin does not have a clathrin-binding domain, it needs an adaptor protein for association with the clathrin coat. Interaction with the proline-rich domain of dynamin is known to be required for its targeting to endocytic sites (Shpetner et al., 1996). Amphiphysin is a very attractive candidate for this function. Amphiphysin is one of dynamin's major interaction partners and is an integral component of the clathrin coat with its SH3 domain accessible for interactions (paper III). When amphiphysin's SH3 domain is overexpressed in fibroblasts (Simpson et al., 1999; Wigge et al., 1997b) or microinjected into lamprey reticulospinal axons (Shupliakov et al., 1997), dynamin fail to oligomerize around the neck of coated pits. This suggests that amphiphysin recruits dynamin to endocytic intermediates *in vivo*. However, some data are in conflict with this notion. Both dynamin and amphiphysin are recruited already to shallow endocytic intermediates (paper II, III, IV) and are implicated in functions at stages prior to fission (Farsad et al., 2003; Narayanan et al., 2005). Moreover, the amphiphysin I knockout mouse is viable and have only a reduced capacity for membrane reuptake in hippocampal neurons (Di Paolo et al., 2002), and fruitfly NMJs do not contain amphiphysin but are able to recycle vesicles efficiently (Leventis et al., 2001; Razzaq et al., 2001; Zelhof et al., 2001). Together these data suggest that although amphiphysin is important for dynamin localization on coated pits it is not the only protein responsible for dynamin recruitment.

Another potential recruiter of dynamin is intersectin. It binds dynamin via three SH3 domains. Microinjection of intersectin antibodies against the SH3 domain cassette, into living lamprey reticulospinal axons (paper IV), resulted in a block of endocytosis and an accumulation of clathrin-coated intermediates at the fission stage. Overexpression of the SH3 domains in fibroblasts or microinjection of SH3C domain into lamprey giant axons blocks endocytosis (paper IV; Simpson et al., 1999). Further, immunolocalization at the ultrastructural level demonstrates that intersectin is recruited to shallow, coated intermediates simultaneously with dynamin (paper IV). Intersectin may interact with clathrin indirectly through its N-terminal interaction with Eps15, which targets intersectin to clathrin-coated intermediates (Hussain et al., 2001). Together this indicates that the interaction between intersectin and dynamin may mediate the recruitment of dynamin to clathrin-coated pits. An increase in dynamin immunoreactivity at endocytic intermediates after disruption of the interaction rather indicates that it may act as a negative regulator of dynamin recruitment from the synaptic vesicle cluster during synaptic activity, where both dynamin and intersectin reside in resting synapses (paper IV). Furthermore, in intersectin/Dap160 mutant flies, lacking both intersectin and amphiphysin, dynamin is still recruited to periactive zones (Koh et al., 2004). Thus, neither amphiphysin nor intersectin is the primary protein responsible for the recruitment of dynamin to the clathrin coat.

Both amphiphysin and intersectin have been shown to regulate the accessibility of their respective SH3 domains by intramolecular inter-

actions (Farsad 2003, Zamianian 2003). Thus, although recruited to the coated pits early, their respective interactions to dynamin may become important during later stages of vesicle recycling (see below; Fig. 3). Other SH3 domain-containing proteins that may function as recruiters are endophilin, cortactin, and SNX9. Overexpression of endophilin's SH3 domain in fibroblasts results in a block of fission (Simpson et al., 1999), whereas microinjection of SH3 domain antibodies in lamprey reticulospinal axons blocks both invagination and fission of clathrin-coated pits (Gad et al., 2000; Ringstad et al., 1999). Whether these effects are due to a block of its interaction with dynamin or synaptojanin, which is also an endophilin binding partner, remains to be clarified. Another protein that is colocalized with dynamin on clathrin-coated intermediates in fibroblasts is cortactin (Merrifield et al., 2005). Although it has not been studied in neuronal cells, perturbation of the cortactin-dynamin 2 interaction in a non-neuronal cell line results in reduced recruitment of dynamin 2 to the Golgi apparatus (Cao et al., 2005). Yet another protein that has been suggested to recruit dynamin to endocytic sites is SNX9 (Lundmark and Carlsson, 2004; Soulet et al., 2005). It is structurally similar to amphiphysin and shares common binding partners (Lundmark and Carlsson, 2002; Lundmark and Carlsson, 2003). It should be noted, however, that knock-down of SNX9 in fibroblasts does not completely inhibit targeting of dynamin to endocytic sites (Lundmark and Carlsson, 2004; Soulet et al., 2005).

Elucidation of protein-protein interactions driving recruitment of endocytic proteins to synaptic periactive zones will require further experiments with the selective disruption of interactions combined with high-resolution immunolocalization and *in vivo* imaging of the proteins.

The role of intersectin in recruitment of dynamin to the fission complex

Recruitment of dynamin to the coat is mediated by an unknown "protein X" and is then transferred to the fission complex. The fission complex, which detaches coated vesicles from the presynaptic membrane, is composed of dynamin and a set of SH3 accessory proteins, like amphiphysin and endophilin (Hinshaw, 2000; Praefcke and McMahon, 2004). The role of intersectin in this process is not clear. It has been suggested to regulate fission of caveolae in non-neuronal cells (Predescu et al., 2003). That prompted us to investigate the role of the dynamin-intersectin interaction in membrane severing.

Perturbation of the dynamin-intersectin interaction in living reticulospinal axons results in an accumulation of constricted collared coated pits (paper IV). The thickness of the collars, representing the fission complex, is remarkably reduced after microinjection of intersectin antibodies as compared to those formed after microinjection of GTPγS (a non-hydrolysable analog of GTP), suggesting that components of the fission complex are missing. Immunolabeling of these structures demonstrate an increase in dynamin labeling, while the level of amphiphysin was not changed significantly (paper IV). Thus, even though excessive amounts of dynamin are present, the fission reaction is still inhibited. This suggests that dynamin is not properly delivered to assemble into a functional fission complex (Fig. 3B; paper IV). We hypothesize that intersectin is important for displacement of dynamin from the coat to the neck of endocytic intermediates. When antibodies block intersectin SH3 domain interactions, dynamin is not recruited in sufficient concentration to form a functional fission complex. The microinjection of SH3C domain most probably interfer at a stage when dynamin is transferred from a recruiting protein X to intersectin or from intersectin to amphiphysin, which eventually prevents clathrin-coated pits from pinching off (Fig. 3C).

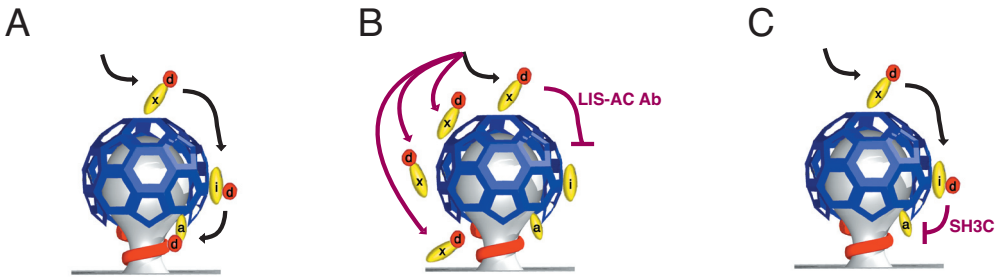

Figure 3: Schematic representation of the sequential steps of dynamin recruitment during formation of the clathrin-coated vesicle in a synapse.
(A) An SH3 domain-containing protein X (yellow) recruits dynamin (d, red) from the synaptic vesicle cluster to the coated pit. Dynamin is then transferred to intersectin (i), and finally delivered to the fission complex (red) via amphiphysin (a). (B) Perturbation of the dynamin-intersectin interaction with intersectin antibodies (LIS-AC Ab) results in recruitment of more dynamin to the coated pit, but the fission reaction is still blocked because dynamin cannot incorporate into the fission complex. (C) Disruption of the dynamin-intersectin interaction with SH3C domain does not prevent normal recruitment of dynamin to coated pits. However, transfer of dynamin from the coat to the fission complex is blocked.

Amphiphysin is recruited to the neck independently of dynamin, since its immunolabeling was not altered to the same extent as dynamin's after microinjection of intersectin antibodies or SH3C domain (paper IV). Perturbation of the interaction between dynamin and amphiphysin completely blocks formation of a fission complex, suggesting that amphiphysin is an important interaction partner for dynamin at this step (Shupliakov et al., 1997; Wigge et al., 1997b). Translocation of dynamin from intersectin to amphiphysin probably occurs prior to fission complex formation. What is the composition of the thin collars observed at coated pits after microinjection of intersectin-perturbing compounds? It is possible that they are composed mainly of amphiphysin and endophilin, which may form spirals *in vitro* (Farsad et al., 2001; Takei et al., 1999). It cannot be excluded that "the intersectin step" can be partially bypassed and recruitment of dynamin to the fission complex may occur without it. Further studies are needed to resolve these issues. Our present results strongly indicate that dynamin is recruited to the neck of coated intermediates via sequential interactions with different SH3 domain-containing proteins located on the clathrin coat prior the fission complex formation, where intersectin is a transit station in this cascade working upstream of amphiphysin (Fig. 3A).

Conclusions and future prospects

Synaptic activity promotes actin filament formation in the periactive zone, which occurs in parallel to dispersion of synapsin into the same synaptic region. Disruption of synapsin function in the living synapse results in a reduction of the actin cytomatrix at sites of synaptic vesicle recycling.

In addition to synapsin, the endocytic proteins dynamin, amphiphysin, and intersectin, are compartmentalized in the synaptic vesicle cluster in central synapses. Synapses established by axons with a tonic activity pattern contain higher levels of these proteins than phasic synapses, which suggest higher efficiency of the endocytic machinery in synapses releasing neurotransmitter at high rates.

Dynamin, amphiphysin, and intersectin are recruited to endocytic intermediates at initial stages of clathrin coat formation and are associated with the coat complex until formation of the fission complex.

Disruption of amphiphysin CLAP-domain interactions by antibodies results in the assembly of clathrin-coated pits with abnormal shapes, which suggests that amphiphysin is integrated in the coat during synaptic vesicle membrane retrieval.

Dynamin is recruited to clathrin-coated pits independently of intersectin and amphiphysin. However, intersectin is important for the recruitment of dynamin to the fission complex where it may act as a transition step upstream of amphiphysin during fission complex formation.

The molecular mechanisms underlying clathrin-mediated endocytosis in central synapses are still waiting to be dissected. While many protein-protein and protein-lipid interactions have been described biochemically for proteins involved in synaptic vesicle trafficking, it is unknown how they are related to the synaptic vesicle cycle. Future studies where specific protein interactions are disrupted and related to molecular and morphological changes in living synapses are required to gain knowledge of the endocytic machinery in synapses. For example, in this thesis I described how the actin nucleating property of synapsin is not likely to be relevant in the synaptic vesicle cluster, but rather in the periactive zone of the synapse. It will also be interesting to address which signals trigger dissociation of endocytic proteins from the synaptic vesicle cluster, and how their targeting to the periactive zone is regulated. Another step of the vesicle cycle that needs to be investigated further is fission. It is still not known how dynamin works and how other proteins in the fission complex contribute to membrane scission. Future studies where the function of endophilin, amphiphysin, and dynamin are disturbed in the fission complex and then analyzed with high-resolution techniques will be important for deciphering how fission works.

Acknowledgements

First of all I would like to thank Oleg Shupliakov for being my supervisor during my PhD studies. You have always given me guidance, encouragement, and utmost support during my studies and challenged me to look at problems from new perspectives.

A big thanks goes out to Nikolay Tomilin for all that you have taught me, your practical support, and contributions to my work. Большое спасибо!

I am also deeply indebted to the other members of the group who have been incredible helpful in discussions, optimizing PCRs, and processing of EM specimens. I really appreciated your positive attitudes, help, and encouragement. Thanks Lennart, Peter, Anna, Kristin, Fredrik, Helge, Joel, Jenny, Victoria, Ylva, Wei, Elena, Staffan, Francisco, Malin, Felicia, and Britt.

I also want to thank my external collaborators: Ona Bloom and Paul Greengard at Rockefeller University, Melissa Marcucci, Vladimir Slepnev, and Pietro De Camilli at Yale University, Tong-Wey Koh and Hugo Bellen at Baylor College of Medicine, Elena Zotova at Albert Einstein College of Medicine, and Simen Gylterud at Oslo University.

Thanks go out to friends and colleagues at the Department of Neuroscience and the Department of Cell and Molecular Biology at Karolinska Institutet.

A special thanks to all my friends in the Credo International Student group for teaching me so much, for your true friendship, and for your prayers.

Jag vill också tacka min vänner Madde, Liselotte, Linda, Olof, Karna, Maria, Daniel, och Maria för att ni inte har slutat höra av er trots att jag har gömt mig nere i källarna på KI de senaste åren.

Till sist vill jag tacka till min familj för det fantastiska stöd som ni har gett mig. Tack mamma och pappa för att ni alltid har uppmuntrat mig och för att ni har lärt mig att våga prova nya vägar. Jag vill tacka mina syskon, Lena, Karin och Lars, för att ni alltid ställer upp i alla lägen. Tack för alla "proffessorskakor", brev, och roliga historier om smågalna kollegor och misslyckade arbetsdagar som har muntrat upp mig.

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