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Role of Alix in endocytosis at presynaptic boutons

Kwangil Chi

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THÈSE

Pour obtenir le grade de

DOCTEUR DE LA COMMUNAUTE UNIVERSITE GRENOBLE ALPES

Spécialité : **Neurosciences, Neurobiologie**

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Présentée par

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Alpes**

préparée au sein du **Grenoble Institut des Neurosciences –
INSERM U1216**

dans **l'École Doctorale de Chimie et Sciences du Vivant**

Rôle d'Alix dans l'endocytose au niveau des boutons pré- synaptiques

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The Role of Alix in Endocytosis at Presynaptic Boutons

Kwangil CHI

**A Thesis submitted for the degree of Doctor of
Philosophy**

January 2019

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List of Abbreviations

ADBE – Activity-Dependent Bulk Endocytosis
Adp1 – Auxin binding protein 1
ADP / ATP – Adenosine DiPhosphate/Adenosine TriPhosphate
AIP1 – Apoptosis Interacting Protein 1
ALG-2 – Apoptosis Linked Gene 2
Alix – ALG-2 Interacting protein X
AMPA(R) – α -Amino-3-hydroxy-5-Methyl-4-isoxazolePropionic Acid (Receptors)
AP180 – Assembly Protein 180
AP2 – Adaptor Protein 2
ARF6 – ADP-Ribosylation Factor 6
Arp2/3 – Actin-related protein 2/3
BAR - Bin-Amphyphysin-Rvs (N-BAR: N-terminal amphipathic helix-BAR / F-BAR: Fes/CIP4 homology-BAR)
BAPTA – 1,2-Bis(o-AminoPhenoxy)ethane-N,N,N',N'-Tetraacetic Acid
CaMK-II – Calcium/CalModulin-dependent protein Kinase II
cAMP – cyclic Adenosine MonoPhosphate
CAZ – Cytomatrix of the Active Zone
CDC42 – Cell Division Control protein 42
CDK5 – Cyclin-Dependent Kinase 5
CEP55 – CEntrosomal Protein 55
CGN – Cerebellar Granule Neurons
CHMP – CHarged Multivesicular body Protein
CME – Clathrin-Mediated Endocytosis
CIE – Clathrin-Independent Endocytosis
CIN85 – Cbl-Interacting Protein of 85kDa
CME – Clathrin-Mediated Endocytosis
CTxB – Cholera Toxin chain B
EGF(R) – Epidermal Growth Factor (Receptor)
EGTA – Ethylene Glycol-bis(β -aminoethyl ether)-N,N,N',N'-Tetraacetic Acid
EIAV – Equine Infectious Anemia Virus
ELKS/CAST – protein rich in the amino acids E,L, K and S/Cytomatrix at the Active zone-Associated
SStructural protein
EPS15 – EPidermal growth factor receptor Substrate 15
EPSC – Excitatory PostSynaptic Current
EPSP – Excitatory PostSynaptic Potential
ESCRT – Endosomal Sorting Complexes Required for Transport
F-actin – Filamentous-actin
FCHO1/2 – Fer/Cip4 Homology domain-Only 1/2
GABA_A(R) – γ -AminoButyric Acid A (Receptor)
G-actin – Globular-actin
GIT – G protein coupled receptor kinase 2 InTeracting protein
GPI – GlycosylPhosphatidyInositol

GSK3 – Glycogen Synthase Kinase 3
GTP – Guanosine TriPhosphate
HDPTP – His Domain PhosphoTyrosine Phosphatase
HIV – Human Immunodeficiency Virus
ILV – IntraLuminal Vesicles
LBPA – LysoBisPhosphatidic Acid
LTD – Long-Term Depression
LTP – Long-Term Potentiation
MEF – Mouse Embryonic Fibroblast
MHCI – major histocompatibility complex class I
MVB – MultiVesicular Bodies
N-WASP – Neuronal Wiskott-Aldrich Syndrome Protein
NMDA(R) – N-Methyl-D-aspartic Acid (Receptor)
PAR1 – Protease Activated Receptor 1
PIP – PhosphatidyInositol Phosphates
PIPky – PhosphatidyInositol Phosphate Kinase type Iy
PI3K – PhosphatidyInositol-3-Kinase
PI(4,5)P₂ – PhosphatidyInositol 4,5-bisPhosphate
PI(3,4,5,)P₃ – PhosphatidyInositol 3,4,5-trisPhosphate
PKA – Protein Kinase A
PPD – Paired Pulse Depression
PPF – Paired Pulse Facilitation
PPR – Paired Pulse Ratio
PRD / PRR – Proline-Rich Domain / Proline-Rich Region
PSD – PostSynaptic Density
PTD – Post Tetanic Depression
PTP – Post Tetanic Facilitation
RIM – Rab3-Interacting Molecules
RIM-BP – RIM-Binding Protein
RRP – Readily Releasable Pool
SH3 – Src Homology 3
SNAP – SyNaptosomal-Associated Protein
SNARE – Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment protein Receptor
STED – STimulated Emission Depletion
SV – Synaptic Vesicle
TNF(R) – Tumour Necrosis Factor (Receptor)
Tsg-101 – Tumour susceptibility gene-101
VAMP – Vesicle-Associated Membrane Protein
VGLUT – Vesicular GLUtamate Transporter
Vps4 – Vacuolar Protein Sorting 4
WT – Wild-Type

Preface

Synaptic vesicle recycling refers to the regeneration of synaptic vesicles from the presynaptic membrane after fusion. The term was coined by J.E. Heuser and T.S. Reese in 1973 through their work on frog neuromuscular junctions (Heuser and Reese, 1973). This electron microscopic study revealed the formation of cisternae derived from the presynaptic membrane after stimulation. The cisternae then divided to form fusion-competent synaptic vesicles. Since then, a number of different modes of synaptic vesicle recycling and the molecules involved have been discovered, that can be categorised as clathrin-mediated endocytosis or clathrin-independent endocytosis. Clathrin-mediated endocytosis is the best understood and it was considered the main route of synaptic vesicle regeneration. The significance of clathrin-independent endocytosis has only recently been brought to light and it is less well understood due to its recent discovery. Therefore, it is vital to identify the molecules involved and their mechanism of action in this process. The present study aims to contribute to the growing knowledge on synaptic vesicle recycling by putting forward Alix (ALG-2 Interacting Protein X) and its interacting proteins (ALG-2 and endophilin) as molecular actors in clathrin-independent endocytosis in synapses.

Introduction

1. Synapses

Neuronal communication is central to the functioning of the central nervous system and underpins its physiological roles such as learning and storing memory. The 100 billion nerve cells that make up the human brain are called neurons, and these are excitable cells that are responsible for transmitting chemical and electrical signals. Neurons are compartmentalised into somato-dendritic compartments, that receive and integrate electrical signals, and axons, where action potentials are generated. Action potentials are temporary shifts in the neuron's membrane potential. Neurons transmit signals between each other through chemical or electrical synapses that are made up of a presynaptic terminal (or bouton) and a postsynaptic terminal. Electrical synapses allow flow of ions and metabolites between neurons through specialised structures called gap junctions. Chemical synapses allow information relay via the release of neurotransmitters from the presynaptic bouton that is detected by receptors on the postsynaptic terminal, and they can be excitatory or inhibitory (Pereda, 2014). In chemical excitatory synapses of the central nervous system, postsynaptic terminals are defined by the presence of postsynaptic densities (PSDs) on the plasma membranes of dendrites whereas presynaptic terminals are often present as boutons in axons. Briefly, synaptic transmission occurs when action potentials depolarise the presynaptic membrane, causing the release of neurotransmitters via synaptic vesicle (SV) fusion to the presynaptic membrane. Neurotransmitters will then diffuse across the synaptic cleft and bind receptors on the postsynaptic membrane to cause depolarisation. Like the PSDs of postsynaptic terminals, there are some defining features of presynaptic boutons and postsynaptic terminals. Together, the presynaptic boutons and postsynaptic terminals form chemical synapses through which neurons communicate.

1.1. Presynaptic Boutons

Presynaptic boutons are specialised parts of the axon that are characterised by the presence of active zones and SVs. This is where the SV fusion occurs to allow the release of neurotransmitters.

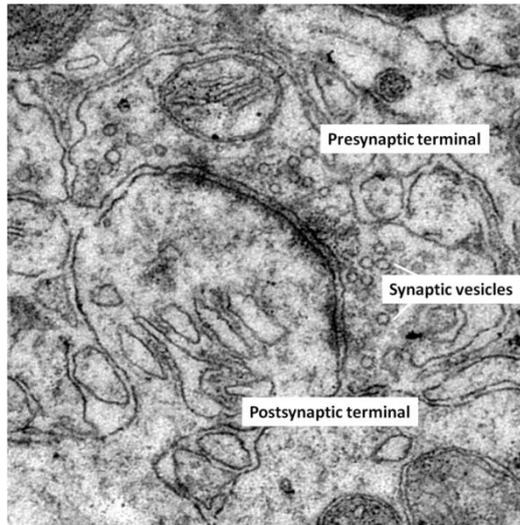
1.1.1. Active Zones

The active zones of presynaptic boutons are electron-dense meshwork of a range of multi-domain proteins which are called the cytomatrix of the active zone, or CAZ, and these are the sites of SV exocytosis (Gundelfinger and Fejtova, 2012). There are five constituent proteins of CAZ that are conserved across organisms (such as flies, rodents and worms) which include ELKS/CAST¹, liprin, Munc13, Rab3-interacting molecules (RIM) and RIM-binding proteins (RIM-BP) (Sudhof, 2012; Michel *et al.*, 2015). CAZ in vertebrates also comprise bassoon and piccolo. These proteins contain protein-protein interaction domains such as C2, PDZ² and Src homology 3 (SH3) domains that allow the tight association of the proteins for regulation of SV fusion to the presynaptic membrane. The protein composition and the relative organisation of CAZ are summarised in figure 1 and table 1. The active zone is encompassed by a periaxial zone, which is where SV endocytosis occurs (Sudhof, 2012). Periaxial zones also house cell-adhesion molecules and presynaptic receptors that modulate neurotransmitter release (e.g. endocannabinoid CB1 receptors). How the different components of the CAZ allow the fusion of SVs for the release of neurotransmitters will be discussed later.

¹ ELKS/CAST: protein rich in the amino acids E, L, K and S/cytomatrix at the active zone-associated structural protein

² named after postsynaptic density protein (PSD-95), discs-large tumor suppressor (Dlg), and tight junction protein-1 (ZO-1)

A



B

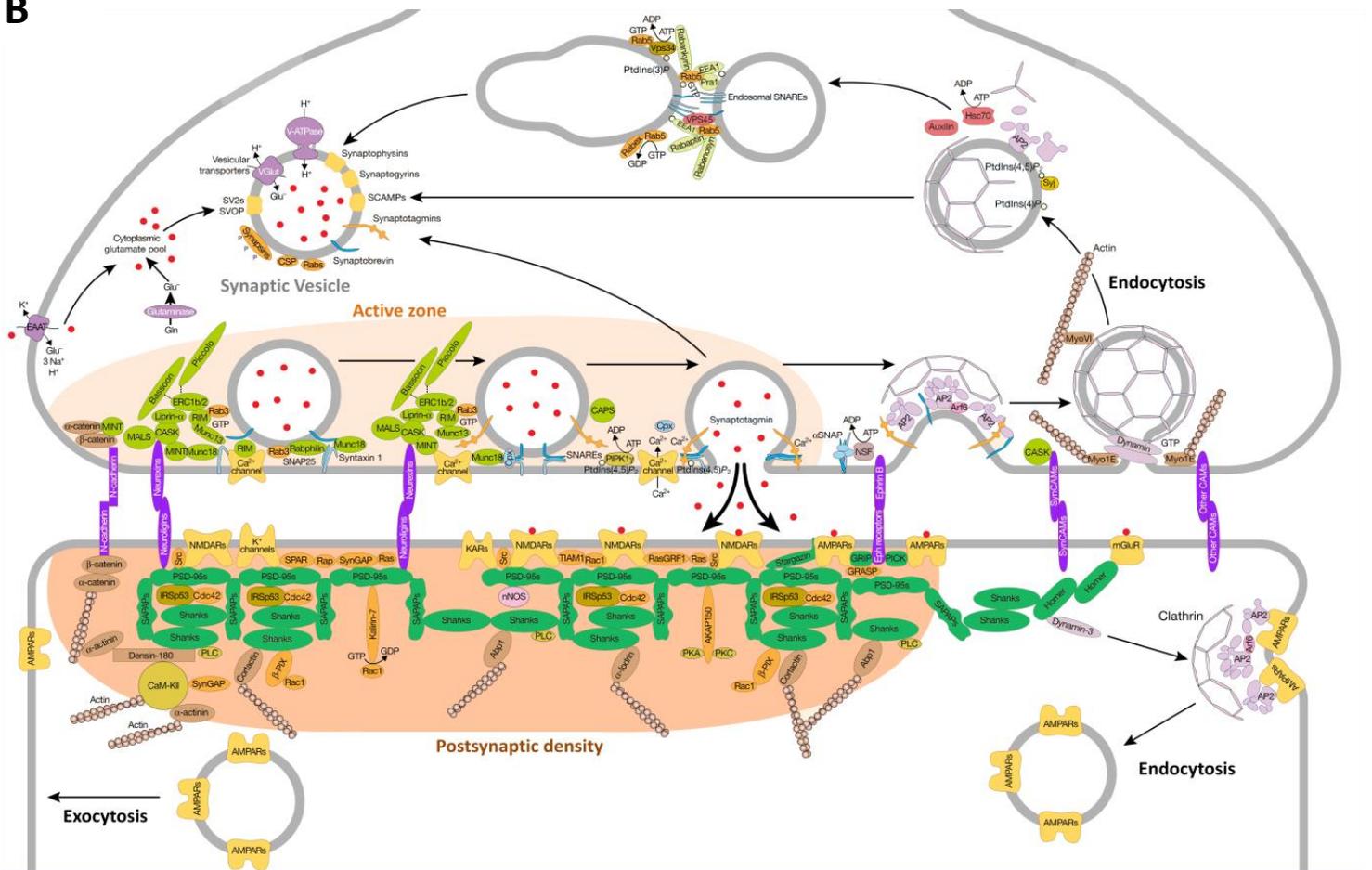


Figure 1: Molecular organisation of a synapse

A) Electron micrograph of a brain slice showing a synapse: a synaptic vesicle-filled presynaptic terminal and a postsynaptic terminal. **B)** A model of the molecular organisation of a synapse.

- Key :
- Adhesion molecules
 - Active zone proteins
 - Endocytic proteins
 - Exocytic proteins
 - Ion channels and glutamate receptors
 - Postsynaptic density proteins

Adapted from (Chua *et al.*, 2010)

| Category | Protein | Function | Interactions |
|---------------------------|--|---|--|
| Active zone proteins | ELKS/CAST | SV exocytosis | Liprin, Rab6 |
| | Liprin | Active zone assembly SV exocytosis | CASK, ELKS, ERC, GIT1, RIM |
| | Munc13 | SV priming | Munc18, RIM, syntaxin |
| | Munc18 | SV priming | Munc13, Syntaxin, VAMP2 |
| | RIM | Ca ²⁺ -channel recruitment SV docking and priming | Ca ²⁺ -channel, ELKS, Munc13, Rab3 (SV), Rab27, RIM-BP |
| | RIM-BP | Ca ²⁺ -channel recruitment | Ca ²⁺ -channel, RIM |
| | CASK | SV recruitment | Liprin, β -Neurexin |
| | Bassoon | SV clustering SV exocytosis | ERC (RIM) |
| | Piccolo | SV clustering | Ca ²⁺ , stonin |
| Exocytic machinery | Ca ²⁺ -channel | Ca ²⁺ entry | RIM, RIM-BP, synaptotagmin |
| | PI(3,4)P ₂ | Synaptotagmin recruitment | Synaptotagmin |
| | SNAP25 | SV fusion | Syntaxin, VAMP2 |
| | Synaptobrevin | SV fusion | Munc18, SNAP25 |
| | Synaptotagmin | SV fusion (Ca ²⁺ sensing) | B-Neurexin, Munc13, PI(3,4)P ₂ |
| | Syntaxin | SV fusion | Munc18, SNAP25 |
| Endocytic machinery (CME) | Amphiphysin | Dynamin recruitment | Dynamin, endophilin, phospholipase D2, synaptojanin |
| | Accessory proteins (AP2, AP180, auxilin, Eps15, epsin, stonin 2) | Cargo recognition and clustering Membrane deformation | Cargo, clathrin, PI(3,4)P ₂ , AP180, AP2, epsin, synaptojanin |
| | Clathrin | Membrane deformation | Amphiphysin, auxilin, AP180, AP2, |
| | Dynamin | Membrane scission | Amphiphysin, PI(3,4)P ₂ , synaptojanin, syndapin |
| | Endophilin | Membrane deformation Synaptojanin recruitment | Amphiphysin, dynamin, synaptojanin |
| | Synaptojanin | Clathrin coat disassembly | Endophilin, PI(3,4)P ₂ |
| Adhesion molecules | Neurexin | SV recruitment AMPA and NMDAR organisation | CASK, synaptotagmin, neuroligin (AMPA, NMDAR) |
| | EphB, Ephrin-B | AMPA and NMDAR trafficking and function | AMPA, NMDAR, PSD-95 |
| | N-cadherin | F-actin organisation | B-catenin |
| | NLG | PSD formation NMDAR clustering | PSD-95 |
| Scaffold proteins | Homer | PSD structure maintenance | Shank |
| | PSD-95 | PSD structure maintenance | Stargazin (AMPA), GKAP, NLG, NMDAR |
| | SAPAP | PSD structure maintenance | PSD-95, Shank |
| | Shank | Links PSD with F-actin | Cortactin, GKAP, homer |
| Glutamate receptors | AMPA | Na ⁺ entry | Stargazin |
| | NMDAR | Na ⁺ and Ca ²⁺ entry | CaMKII, PSD-95 |

Table 1: A summary of the main proteins comprising a synapse.

1.1.2. Synaptic Vesicles and Synaptic Vesicle Pools

SVs are storehouses of neurotransmitters that are uniform in size with diameters of around 30 to 50 nm (Poudel and Bai, 2014). It is suggested that these uniform SVs also contain approximately the same amount of neurotransmitters which is the basis of reliable quantal release of neurotransmitters. They are compact structures with over 60% of their total mass coming from proteins alone. It is estimated that up to 200 protein molecules are present in a single SV. The protein composition of a typical SV is summarised in table 2 (Südhof, 1999; Wilhelm *et al.*, 2014). Different synapses have varying numbers of SVs, for example, rodent hippocampal synapses typically have around 200 SVs whereas the calyx of Held around 300 SVs (Gan and Watanabe, 2018). Such a difference reflects the physiological requirements of these synapses. Compared to the relatively small hippocampal synapses, the calyx of Held, found in the auditory nervous system, is a large synapse which is often subject to very high firing rates³ that can reach up to around 1 kHz, and therefore requires more SVs (Baydyuk, Xu and Wu, 2016; Gan and Watanabe, 2018). Despite the uniformity in size, SVs show heterogeneity in terms of function and organisation (Crawford and Kavalali, 2015) and these differences helped categorise SVs into different groups known as SV pools. These pools are readily releasable pool, recycling pool, total recycling pool and resting pool (Alabi and Tsien, 2012; Fowler and Staras, 2015) and are summarised in figure 2.

SVs of the readily releasable pool are the first to be released upon stimulation. In hippocampal synapses, the readily releasable pool accounts for around 10 SVs (Gan and Watanabe, 2018). These are typically the SVs that are docked to the active zone, ready for release. It is generally considered that the readily releasable pool depletion can be achieved by various stimulation conditions such as a few seconds of hypertonic shock or tens of action potentials at mild frequencies (Rizzoli and Betz, 2005; Fowler and Staras, 2015). The replenishment of the readily releasable pool takes less than 30 seconds to complete in hippocampal synapses (Goda and Stevens, 1998).

³ Firing rate refers to the average number of action potentials per unit time

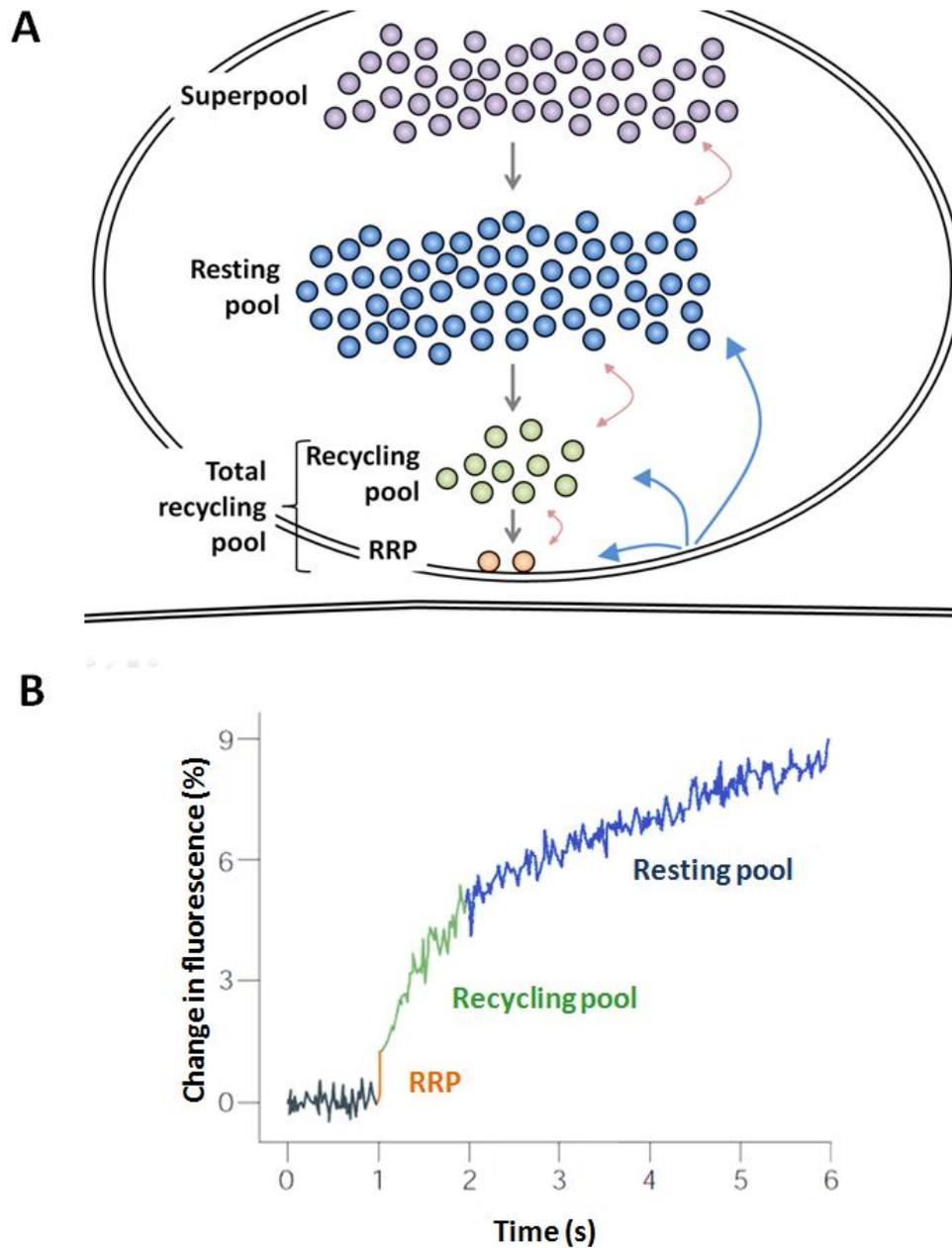


Figure 2: synaptic vesicle pools

A) The four different synaptic vesicle pools present in a presynaptic bouton. The readily releasable pool (RRP) represents the docked synaptic vesicles that are primed for release. The recycling pool accounts for around 5~20% of all synaptic vesicles, that will release immediately after the RRP. Together, the RRP and the recycling pool are called the total recycling pool. The resting pool accounts for around 15~85% of all synaptic vesicles, that are reluctant to release. The superpool represents the synaptic vesicles that are transported along axons to different presynaptic boutons, likely for the sharing of synaptic vesicles where necessary. **B)** the kinetics of release of the various synaptic vesicle pools. Goldfish bipolar cells were stimulated in the presence of FM1-43, a styryl dye that fluoresces upon integration into membranes. Three kinetic components of exocytosis were reported that were suggested to derive from different synaptic vesicle pools (indicated on the graph). **A)** and **B)** are adapted from (Rizzoli and Betz, 2005)

| Category | Protein | Description | Average Copy Number /Vesicle |
|---------------------------|-----------------|---|------------------------------|
| Synaptic vesicle proteins | Proton ATPase | Pumps protons into SVs | 1.27 |
| | SV2 | Function uncertain | 5.04 |
| | Synaptogyrin-1 | Implicated in SV recycling and synaptic plasticity | 6.80 |
| | Synaptophysin | Implicated in synapse formation, SV docking, SV endocytosis | 13.06 |
| | Synaptotagmin-1 | SV fusion (Ca ²⁺ sensing) | 7.16 |
| | VAMP2 | SV fusion | 10.53 |
| | VGLUT | Fills SVs with glutamate | 4.09 |

Table 2: Seven major synaptic vesicle proteins and their corresponding copy number

Adapted from (Mutch *et al.*, 2011)

The release of the recycling pool then follows. The recycling pool accounts for around 16 SVs in hippocampal synapses (Gan and Watanabe, 2018) or 5~20% of all vesicles generally (Rizzoli and Betz, 2005). This pool replenishes the readily releasable pool during mild stimulation conditions and maintains steady release (Alabi and Tsien, 2012). Together the readily releasable pool and recycling pool are known as the total recycling pool which represents the SVs that undergo SV recycling during stimulation conditions within physiological boundaries.

The remaining SVs are grouped in the resting pool that is reluctant to release. The size of resting pool in hippocampal synapses are estimated to be around 200 SVs (Gan and Watanabe, 2018), or around 15% to 85% generally (Fowler and Staras, 2015). It was shown that the resting pool could be released in an extreme condition when the total recycling pool has been depleted as a study on the temperature sensitive-mutant of *Drosophila*, *shibire*, showed (Kuromi and Kidokoro, 1998). The *shibire* gene encodes dynamin in flies and the *shibire* mutation was found to cause mutation in the GTPase domain of this protein (Van Der Bliet and Meyerowitz, 1991). In *shibire*-mutation carrying *Drosophila*, SV endocytosis is inhibited at 'non-permissive temperature' leading to the depletion of the total recycling pool at synapses (Kuromi and Kidokoro, 1998). In this condition, release of the resting pool could be observed indicating that the resting pool could be released once the total recycling pool has been depleted. The physiological role of the resting pool, however, still remains vague, but it appears that SVs are exchanged with the total recycling pool (Fowler and Staras, 2015).

Certain conditions or factors seem to promote the transfer of SVs from the resting pool to the total recycling pool such as cyclin-dependent kinase 5 (CDK5) activity (Kim and Ryan, 2010) or the activation of N-methyl-D-aspartic acid receptor (NMDAR; Ratnayaka *et al.*, 2012). The transfer of SVs from the total recycling pool to the resting pool has been suggested with an antidepressant (Jung *et al.*, 2017) or during calcineurin activity (Marra *et al.*, 2012). These studies suggest SVs are not fixed to a specific pool, but that they could shift from a pool to another.

In addition to these pools, a new pool, termed the superpool has been identified. The superpool is transported along the axon, residing transiently in presynaptic boutons, and allows sharing of SVs across boutons (Fowler and Staras, 2015). In a study on rat hippocampal neurons, it was shown that newly recycled SV vesicles from one bouton can be transferred to a neighbouring bouton to be released in a subsequent stimulation (Darcy *et al.*, 2006). This indicates that the superpool is a mix of SVs from the total recycling pool. The transport of superpools between presynaptic boutons may be active, along the cytoskeleton, or passive, by free diffusion (Westphal *et al.*, 2008). It is suggested that certain factors regulate the superpool and that the function of this pool might be to provide an extra supply of SVs where necessary (Staras and Branco, 2010). This further supports the idea that various SV pools are intermixable.

In terms of the organisation within presynaptic boutons, the total recycling pools are generally found closer to sites of SV fusion and the resting pools are found distant from such sites (Fowler and Staras, 2015). It has been suggested that various molecular markers help SV pool organisation by differentially associating with the different SV pools. For example, vesicle-associated membrane protein 7 (VAMP7) appeared to be enriched in SVs of the resting pool, and these SVs were distinct from those enriched in VAMP2 and vesicular glutamate transporter-1 (VGLUT-1) that represented the recycling pool (Hua *et al.*, 2011). Synapsin is a protein that has been implicated as a molecular glue that binds the resting pool together, forming boundaries and limiting lateral leakage of SVs into axons (Orenbuch *et al.*, 2012). Altogether, the dynamic nature of SVs and their highly-regulated organisation allow distinct pools of SVs that can intermix in adaptation to changes at synapses.

1.2. Postsynaptic Terminals

Postsynaptic terminals are specialised structures on dendrites and the molecular organisations can differ depending on the nature of the synapse, either excitatory or inhibitory (Sheng and Kim, 2011). For the purpose of this thesis, only excitatory synapses will be discussed. PSDs are electron-dense regions of the dendritic spines harbouring a range of proteins including calcium/calmodulin-dependent protein kinase II (CaMK-II), glutamate receptors, PSD-95, Shank, and components of the actin cytoskeleton. Other proteins of the postsynaptic terminal and their relative organisation are shown in figure 1 and table 1.

Postsynaptic terminals and presynaptic boutons are held together by a number of adhesion molecules that are in the synaptic cleft including integrins, neuroligins and ephrin receptors (Harris and Weinberg, 2012). In addition to the adhesion function, some of these proteins have roles in synaptogenesis and synaptic plasticity.

1.3. Synaptic Transmission

Action potentials arriving at the presynaptic terminal (or bouton) cause depolarisation. This triggers the opening of voltage-gated calcium channels which leads to the fusion of neurotransmitter-filled synaptic vesicles (SVs) to the presynaptic membrane in full-collapse fusion, namely exocytosis. Then compensatory endocytosis will reform SVs by pinching off a part of the presynaptic membrane and replenish the SV pool. This process is termed SV recycling (Heuser and Reese, 1973) and it is at the core of neurotransmission to allow maintenance of efficient neuronal communication in the dynamic environment of synapses.

1.3.1. Kiss-and-Run

Since the first discovery of SV recycling, it has been theorised that some SVs might not undergo full-collapse fusion, but rather retain their SV identity through transient fusion called 'kiss-and-run' – a term which was first coined by Fesce and his colleagues in 1994 (Ceccarelli et al., 1973; Heuser and Reese, 1973; Fesce *et al.*, 1994). In this mode of SV

recycling, SVs fuse transiently with the presynaptic membrane, allowing a partial discharge of neurotransmitters through the fusion pore before the retrieval of the SV through fusion pore closure. Initial speculations were generally based on morphological observations such as the predominance of vesicle openings in active zones even during the recovery phase (endocytic phase; Fesce *et al.*, 1994). A number of evidences in support of the existence of kiss-and-run have since been brought forward. These include capacitance⁴ flickers that correspond to rapid opening and closing of fusion pores with diameters of >2.3nm (He *et al.*, 2006), partial release of styryl dyes (Aravanis, Pyle and Tsien, 2003), and the retention of 15nm quantum dots during cycles of neurotransmission (Zhang, Li and Tsien, 2009). Such studies do not neglect the existence of other modes of endocytosis. No mechanisms have been shown regarding kiss-and-run at synapses, but suggestions have been made which include a special organisation of the active zone to allow a limited number of full-collapse fusions, and a possible existence of a ring at the neck to restrict full-collapse fusion (Saheki and De Camilli, 2012). A number of studies provide evidence against the presence of kiss-and-run. It was demonstrated that prolonged stimulation of cultured hippocampal neurons at 10Hz (a condition which does not deplete the recycling pool of SVs) leads to an efficient release of FM4-64, suggesting full-collapse fusion events (Fernández-Alfonso and Ryan, 2004). Additionally, it was found that SV proteins that are exocytosed are not always the same as those which are subsequently endocytosed (Fernández-Alfonso *et al.*, 2006; Wienisch and Klingauf, 2006). This implies that SVs undergo full-collapse fusion before a new SV is reformed through compensatory endocytosis, arguing against the existence of kiss-and-run.

⁴ Membranes act as an insulator separating two electrolytic media (the cytoplasm and extracellular environment), resulting in membrane capacitance. The addition of membrane by exocytosis will lead to increased capacitance whereas the deduction of membrane by endocytosis will lead to decreased capacitance.

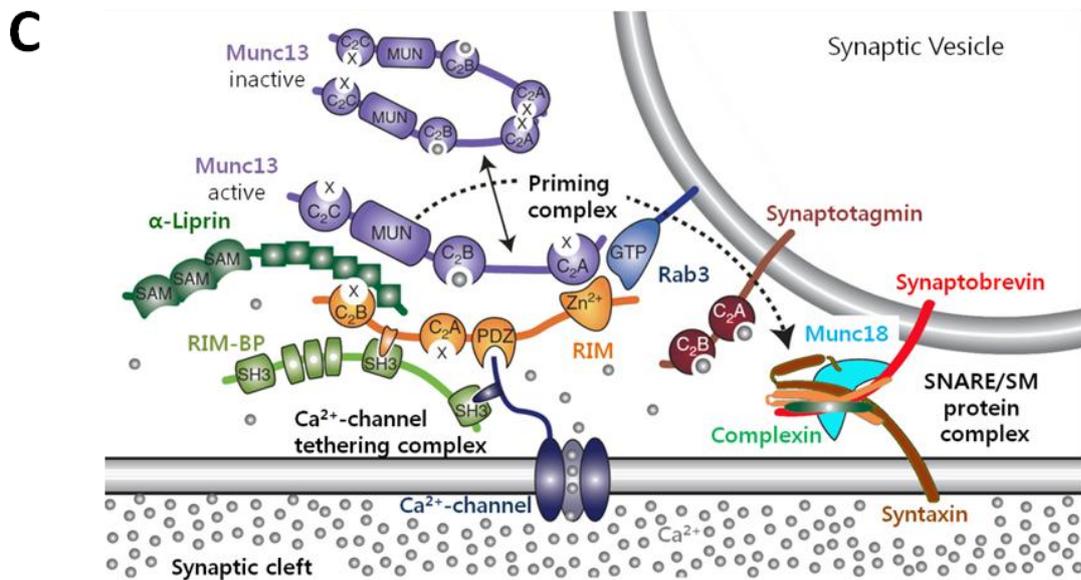
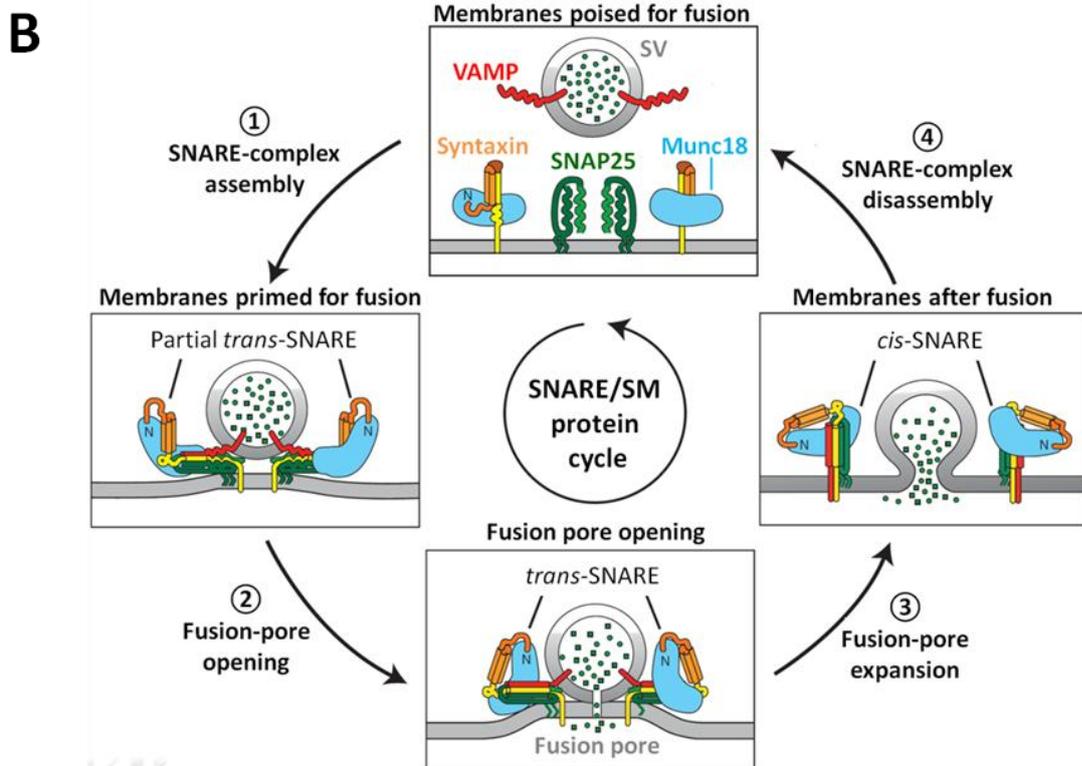
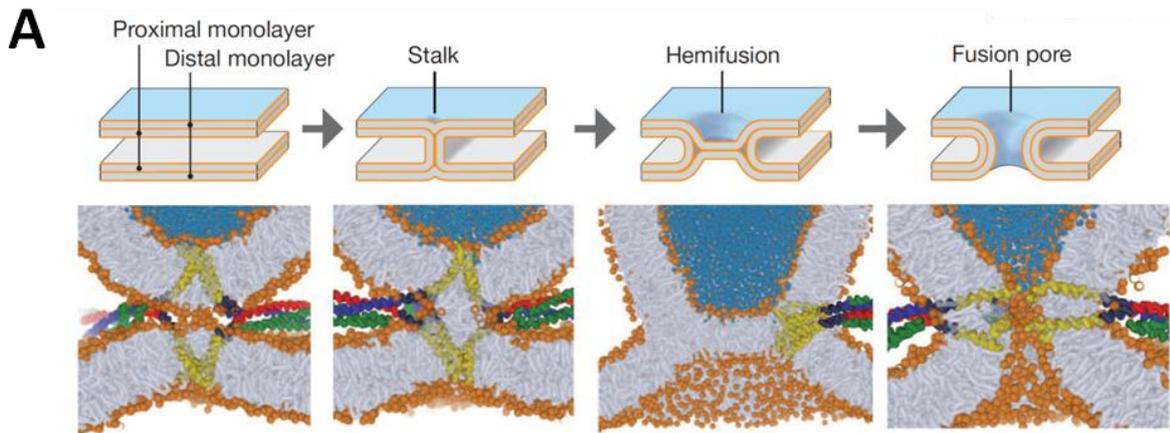


Figure 3: Synaptic vesicle exocytosis (↑)

A) Intermediary steps in SNARE-mediated membrane vesicle fusion. Adapted from (Jahn and Fasshauer, 2012). **B)** Four steps of the SNARE/SM protein cycle. The top diagram shows VAMP (v-SNARE) in its native, unfolded state, syntaxin-1 (t-SNARE) in its closed conformation and bound to Munc18-1 (SM protein), and SNAP-25 (t-SNARE). Syntaxin-1 and SNAP-25 can form heterodimers. The diagram on the left shows membranes primed for fusion. The priming process involves the formation of *trans*-SNARE complexes: the coiled coil interaction between v- and t-SNAREs. The bottom diagram shows the formation of a fusion pore induced by the formation of full *trans*-SNARE complexes. The *trans*-SNARE complexes convert into *cis*-SNARE complexes. The *cis*-SNARE complexes are disassembled by the action of ATPase NSF and its adaptors $\alpha/\beta/\gamma$ -SNAPs, preparing the SNARE/SM proteins for more synaptic vesicle exocytosis. **C)** Active zone proteins during synaptic vesicle exocytosis. Four components of the CAZ (α -Liprins, Munc13s, RIMs and RIM-BPs) are depicted in this diagram. The interaction between the various active zone proteins are mediated by the C2-/SH3-/zinc finger domains. The MUN domain of Munc13 cooperates with Munc18 to allow the correct formation of *trans*-SNARE complex (Lai *et al.*, 2017). **B)** and **C)** are adapted from (Südhof and Rizo, 2011)

1.3.2. Exocytosis

As mentioned earlier, neurotransmitter release or SV fusion occurs at active zones. Synapses of the central nervous system are smaller than other synapses, with only one active zone per synapse in the case of hippocampal synapses (Gan and Watanabe, 2018). SV fusion is a very rapid process, taking only around 200 μ sec, due to the tight connection between the molecular actors involved (Adler *et al.*, 1991).

The fusion and merging of two bilayers into one requires a substantial amount of energy as there are factors that have to be overcome: the electrostatic repulsion by negative charges on the surfaces of the bilayers, and the dehydration barrier. As storage compartments of neurotransmitters, SVs are an important part of neurotransmission and harbour a vast range of proteins and lipids including the 'soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor' (SNARE) complex proteins (Takamori *et al.*, 2006). SNAREs are capable of providing the energy to overcome the unfavourable fusion process. They can be grouped into two types⁵: v-SNAREs that are on SVs and t-SNAREs that

⁵ t-SNARE: target SNARE; v-SNARE: vesicular SNARE; q-SNARE: SNAREs with Gln/Q residue; r-SNARE: SNAREs with Arg/R residue (Hong, 2005)

are on the presynaptic membrane; or alternatively R-SNAREs or Q-SNAREs, respectively (Hong, 2005). Synaptobrevin or VAMP-2 is a v-SNARE, and syntaxin and synaptosomal-associated protein 25 (SNAP-25) are t-SNAREs (Südhof and Rizo, 2011). Other proteins such as the calcium-sensing protein, synaptotagmin, are also involved. Initially SVs will be tethered and primed for exocytosis at the active zone, through a process which is carried out by certain Rab family of small GTPases and their effector proteins. At this point the v-SNAREs and t-SNAREs will interact to form a trans-SNARE complex bringing the SV membrane in tight apposition with the presynaptic membrane. Complexin plays a role in facilitating the formation of this complex, but prevents further zippering (Li *et al.*, 2011). This leaves SVs in a primed state until action potentials induce the entry of calcium through voltage-gated calcium channels in the active zones. Synaptotagmins are SV proteins that harbour two C2 domains (C2A and C2B) that are capable of binding calcium, membrane and SNARE complexes (Yoshihara and Littleton, 2002; Jahn and Fasshauer, 2012). Upon binding calcium, synaptotagmins allow the fusion of SVs to the presynaptic membrane by interacting with the SNARE complex to relieve the inhibitory effect of complexin (Schaub *et al.*, 2006), and initialise and stabilise fusion pores (Yoshihara and Littleton, 2002). It was proposed that the C2B domain of synaptotagmin-1, which interacts with the trans-SNARE complex as well as the plasma membrane, is essential in calcium-dependent SV fusion by deforming the plasma membrane (Wang *et al.*, 2016). The capacities of the C2B domain to deform the membrane and to displace the inhibitory complexin allow the two apposed membranes to come closer together, which leads to the formation of fusion pores. The various steps in the SV fusion process, the SNARE/SM (Sec1/Munc18-like protein) protein cycle and the involvement of the active zone proteins in SV exocytosis are summarised in figure 3.

Components of the CAZ also play a role in SV exocytosis. It was demonstrated that RIMs allow the clustering of calcium channels to the active zone either by direct interaction or through its interaction with RIM-BP (Kaeser *et al.*, 2011). The depletion of RIMs led to reduced calcium influx at presynaptic boutons and a concomitant decrease in neurotransmitter release, suggesting that calcium channel recruitment to active zone mediated by RIMs has a central role in SV exocytosis (Han *et al.*, 2011; Kaeser *et al.*, 2011). Furthermore, an electron tomography study showed that knockout of an isoform of RIM, RIM1 α , caused defects in SV tethering to the active zone and reduced the readily releasable

pool size (Fernández-Busnadiego *et al.*, 2013). This suggested that RIM is necessary for tethering SVs to the active zone.

Munc13 of the CAZ has also been implicated in SV exocytosis. Depletion of an isoform, Munc13-1, in mice led to a significant decrease in the size of readily releasable pool which was accompanied by a blockage of neurotransmitter release at excitatory glutamatergic synapses (Augustin *et al.*, 1999). Furthermore, a double-knockout of Munc13-1 and Munc13-2 in mice resulted in a complete blockage of SV exocytosis in both excitatory glutamatergic synapses and inhibitory, γ -aminobutyrate (GABAergic), synapses (Varoqueaux *et al.*, 2002). Munc13, through its MUN domain, plays a role in SV exocytosis by accelerating the transition of the closed Munc18-syntaxin complex into the trans-SNARE complex (Ma *et al.*, 2011). A recent study revealed another role of the MUN domain of Munc13 in the regulation of SV exocytosis by ensuring proper assembly of the trans-SNARE complex by cooperating with Munc18 (Lai *et al.*, 2017). Such proper assembly of the trans-SNARE complex allows the interaction of the complex with complexin-1 and synaptotagmin-1 to favour calcium-triggered vesicle fusion. In addition, it was shown that Munc13 and RIM are able to form tripartite complexes along with a Ras-related protein, Rab3 (a GTP-binding protein), that facilitate SV priming (Dulubova *et al.*, 2005).

1.3.3. Endocytosis

Endocytosis refers to a set of highly regulated cellular processes that allow the uptake of extracellular material by capturing a part of the plasma membrane. The role of endocytosis can vary from the internalisation of receptors for signalling, to the regulation of the plasma membrane composition. The ingested materials are contained in membrane-bound endocytic vesicles and can either be recycled back to the plasma membrane or degraded in specialised compartments. Such pathways can be hijacked by pathogens to spread disease, or exploited by therapeutic interventions to ameliorate diseases or disorders (Pelkmans and Helenius, 2003). In general, endocytosis is mediated by various molecular actors that act to curve the plasma membrane inward to form invaginations which will be cut into vesicles by scission (Doherty and McMahon, 2009). Endocytosis can be classified into different categories according to the molecular actors involved, as summarised in figure 4.

The well-understood clathrin-mediated endocytosis (CME) involves the coat protein, clathrin, and its adaptor proteins to internalise essential nutrients (e.g. iron) and receptors (e.g. fibroblast growth factor receptor and G-protein coupled receptors; Le Roy and Wrana, 2005; Mukherjee, Tessema and Wandinger-Ness, 2006). Clathrin-independent endocytosis, on the other hand, as the name suggests, does not involve clathrin and is not so well understood. It encompasses a range of endocytic mechanisms such as ADP-ribosylation factor 6 (ARF6)-mediated and cell division control protein 42 (CDC42)-mediated pathways (Sabharanjak *et al.*, 2002; Kirkham and Parton, 2005; Kirkham *et al.*, 2005; Mayor *et al.*, 2014).

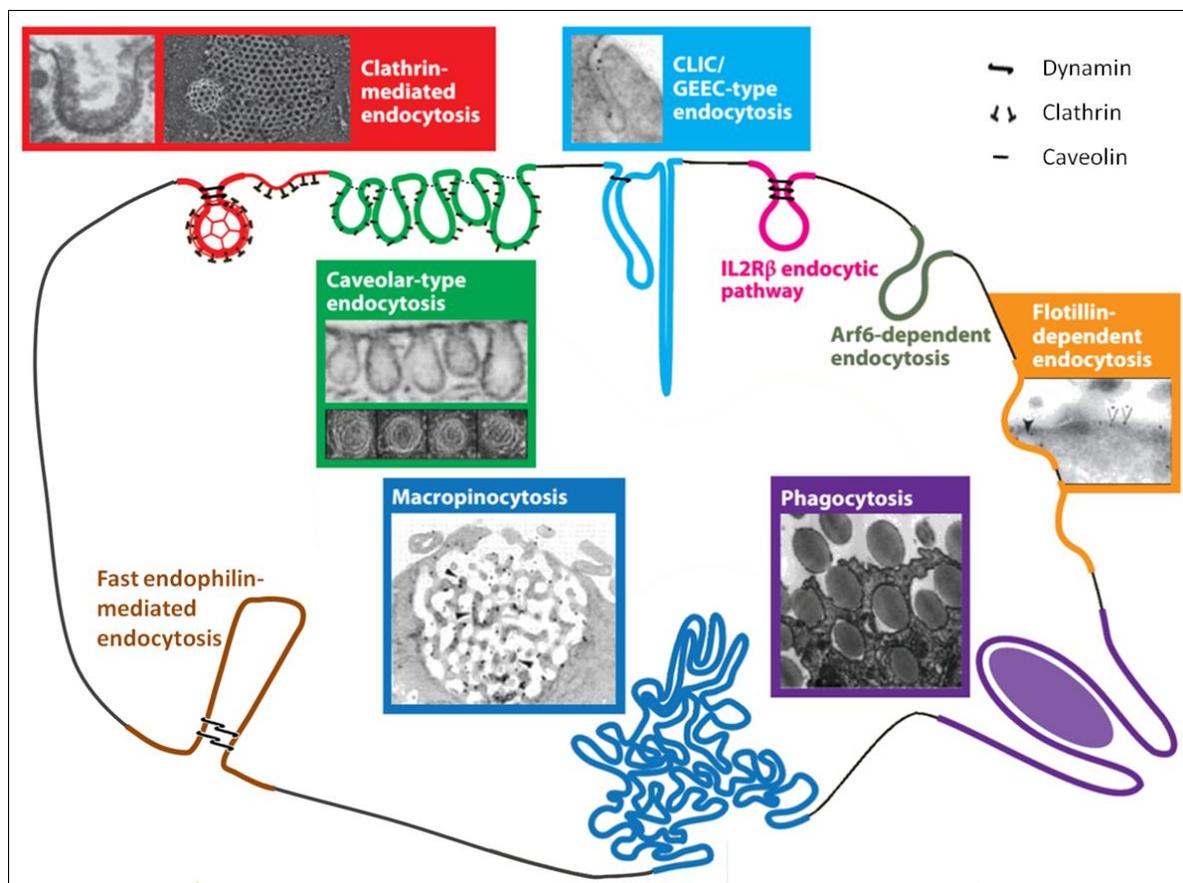


Figure 4: Different types of endocytosis

A range of different endocytic modes can be found in cells. The various modes utilise different proteins to take up different cargoes and varying amounts of the plasma membrane. The nomenclature depends on the main proteins involved or the cargo taken up. For example, clathrin-mediated endocytosis and flotillin-dependent endocytosis utilise clathrin and flotillin, respectively. CLIC/GEEC stands for clathrin-independent carriers/ glycosylphosphatidylinositol-anchored protein enriched endocytic compartments. For more details of each pathway, see (Doherty and McMahon, 2009). Many forms of clathrin-independent endocytosis exist and are not as well-understood as the clathrin-mediated endocytosis. Adapted from (Doherty and McMahon, 2009).

1.3.3.1. Membrane Deformation in Endocytosis

Deforming or re-shaping a flat membrane to form vesicles requires energy due to the propensity of the membrane to remain flat (Hurley *et al.*, 2010). In endocytosis positive membrane curvature is induced which means that the membrane bends inwards, towards the cytoplasm. Negative membrane curvature, which is the outward bending of the membrane, away from the cytoplasm, is induced in processes such as viral budding and exosomes generation. Positive membrane curvature can occur through a number of different mechanisms as summarised in figure 5.

Among the different way to induce positive membrane curvature, protein shape-induced membrane bending is of particular interest to the present study. Bin-Amphiphysin-Rvs (BAR) domain proteins such as endophilin and amphiphysin comprise curved and positively charged BAR domains that are able to bind the negatively charged polar heads of lipids (Peter *et al.*, 2004). The BAR domain superfamily comprises a number of families including the F-BAR (Fes/CIP4 homology-BAR), the N-BAR (N-terminal amphipathic helix-BAR) and others that are reviewed in Frost *et al.*, 2009. A majority of BAR domain proteins have protein-to-protein interaction domains like the SH3 domain (Stanishneva-Konovalova *et al.*, 2016). BAR domain proteins may function by inducing membrane curvature. In support, certain BAR domain proteins such as endophilin and amphiphysin harbour amphipathic α -helices that insert into the lipid bilayers that may facilitate membrane curvature (Frost *et al.*, 2009). Also supporting the idea, a study on large synthetic lipid vesicles called giant unilamellar vesicles showed that amphiphysin acts as curvature sensing modules at low protein density, but acts as curvature-inducing modules at high density (Sorre *et al.*, 2012). An ensuing study reported a similar finding with endophilins suggesting that at high protein density, endophilin also acts to form tubules on giant unilamellar vesicles (Shi and Baumgart, 2015).

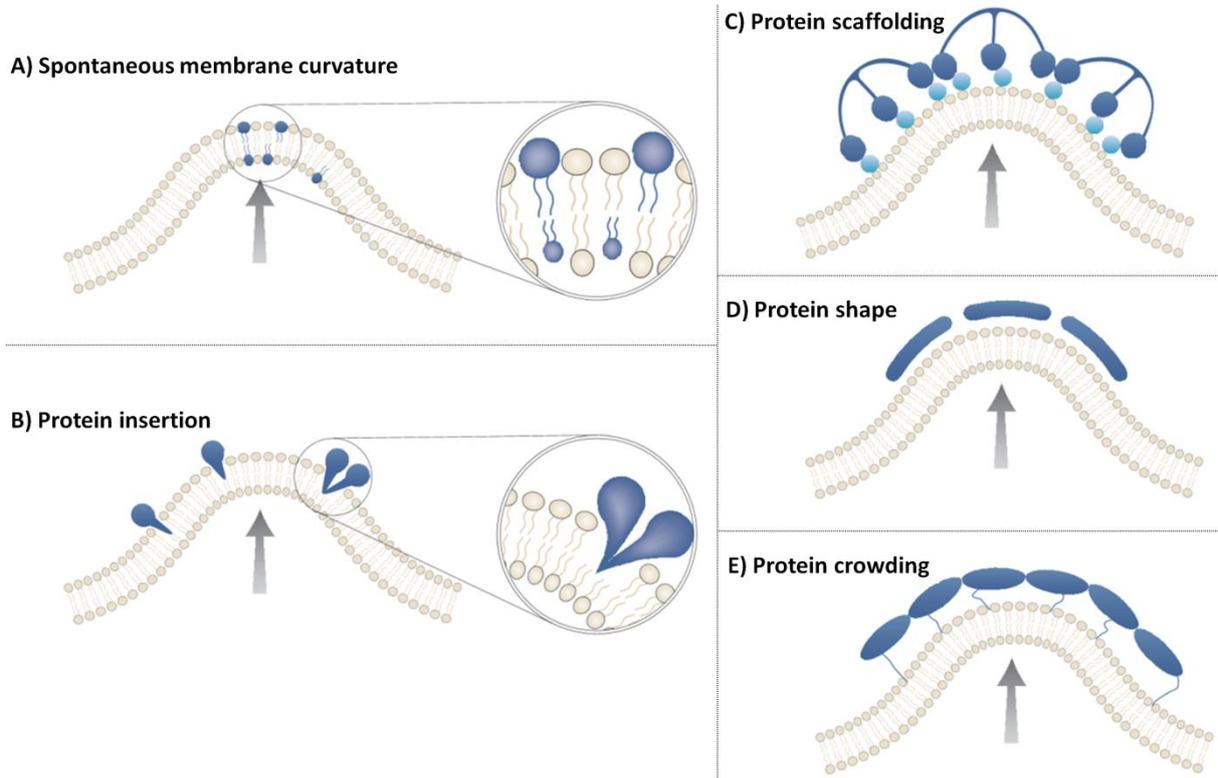


Figure 5: Positive membrane curvature inducing mechanisms.

A) Lipid-induced membrane curvature arising from the presence of lipids with varied polar head groups to acyl chains ratios. **B)** Membrane curvature induced by the insertion of hydrophobic or amphipathic proteins. **C)** Protein assembly-induced membrane curvature, where adaptor proteins anchored to the membrane are pulled by coat proteins such as clathrin. **D)** High concentrations of proteins with curved domains that are positively charged (e.g. BAR domains) alone are enough to generate membrane curvatures and invaginations. **E)** Crowding of surface density proteins can induce membrane curvature. Adapted from (Kirchhausen, 2012).

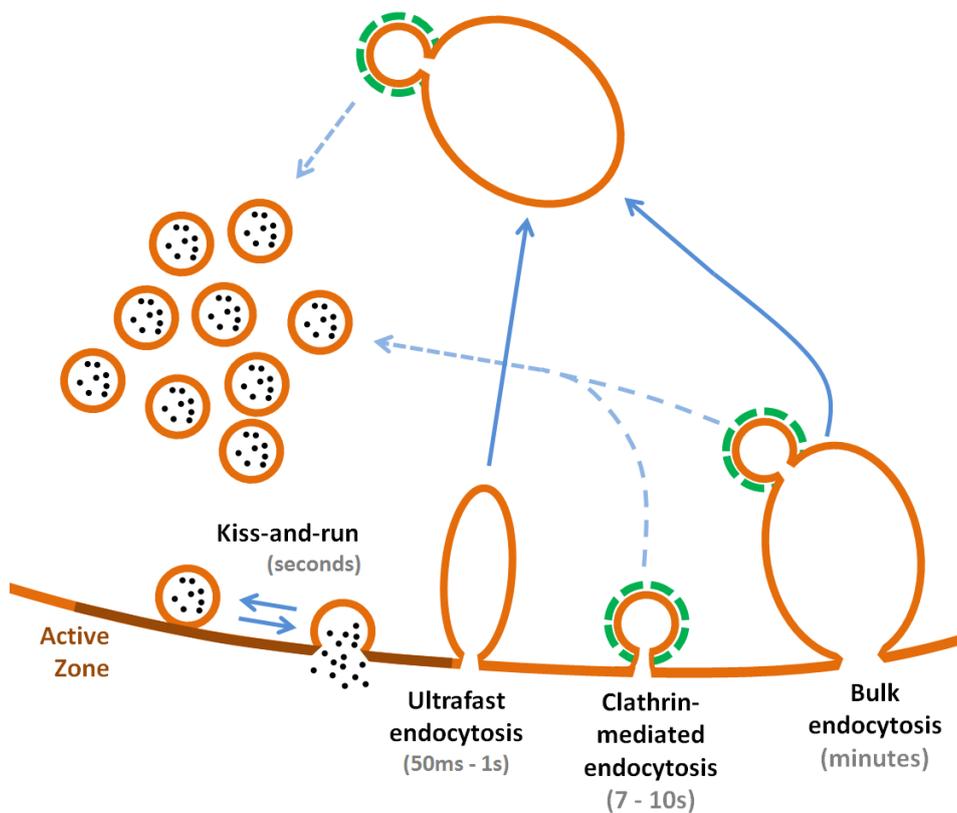


Figure 6: Synaptic vesicle recycling

During synaptic activity, synaptic vesicles fuse with the presynaptic membrane at the active zone. Approximate timings are indicated in brackets below. It was suggested that vesicles may fuse transiently, partially releasing neurotransmitters, before being retrieved through kiss-and-run. Ultrafast endocytosis occurs immediately after fusion, at sites adjacent to active zones and takes up large volumes of membrane (equivalent to approximately four synaptic vesicles). Clathrin-mediated endocytosis, which is the main form of endocytosis during mild activity, takes up single synaptic vesicles in a relatively slow process involving clathrin (green dotted lines) and its adaptors. Bulk endocytosis (or activity-dependent bulk endocytosis), the main form of endocytosis during intense or high frequency activity, takes up bulks of membrane from which synaptic vesicles can be regenerated in a clathrin-dependent manner.

1.3.4. Endocytosis in Presynaptic Boutons

At synapses, compensatory endocytosis ensures efficient neurotransmission by avoiding swelling of the presynaptic membrane and replenishing the limited pool of synaptic vesicles. Initially it was suggested that newly formed vesicles fuse with endosome-like structures before new SVs are generated (Heuser and Reese, 1973). An ensuing study added to this classical model of clathrin-mediated endocytic recycling and proposed that CME can derive single SVs directly from the presynaptic membrane (Takei *et al.*, 1996). The discovery of other modes of endocytosis at presynaptic boutons then followed. Action potential firing rates in hippocampal neurons tend to remain low (up to around 10Hz), but in times of high activity can reach up to 100 Hz (Gan and Watanabe, 2018). SV fusion is a rapid process (around 200 μ sec) and CME is a relatively slow process with a time constant of around 7 to 10 seconds (Adler *et al.*, 1991; Clayton and Cousin, 2009b; Gan and Watanabe, 2018). Considering these facts, it is clear that other modes of endocytosis exist in synapses. A few different forms of CIE have been identified in synapses that led to a deeper understanding of the complex nature of SV recycling. The various modes of SV recycling or endocytosis in presynaptic boutons are summarised in figure 6.

1.3.4.1. Clathrin-Mediated Endocytosis

The initiation of CME is mediated by a pioneer module which comprises the adaptor proteins Fer/Cip4 homology domain-only 1/2 (FCHO1/2), epidermal growth factor receptor substrate 15 (EPS15) and intersectin (Henne *et al.*, 2010; Kaksonen and Roux, 2018). FCHO1/2 interact with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) and mediate the recruitment of other endocytic proteins including adaptor protein 2 (AP2), stonin 2 and assembly protein 180 (AP180). Such adaptor proteins recognise the cytosolic domains of various cargo molecules such as VGLUT-1, synaptotagmin and VAMP2 (Diril *et al.*, 2006; Voglmaier *et al.*, 2006; Koo *et al.*, 2011) allowing eventual recapture of these SV proteins through CME. Clathrin light and heavy chains are then recruited in the form of triskelia that polymerise into the characteristic lattice, deforming and invaginating the membrane, forming clathrin-coated pits (Brodsky *et al.*, 2001). This is depicted in figure 7. The adaptor proteins then recruit BAR domain proteins like amphiphysin and endophilins that are

capable of recognising and attaching to curvatures in the membrane. Although BAR domain proteins are also capable of deforming the membrane, such a function has not been described in CMEs, rather, these BAR domain proteins recruit other proteins like the GTPase dynamin and polyphosphoinositide phosphatase synaptojanin-1 (Di Paolo *et al.*, 2002; Schuske *et al.*, 2003; Verstreken *et al.*, 2003). The GTPase dynamin performs the pinching (fission) process by forming a spiral around the necks of clathrin coated pits that constrict by the hydrolysis of GTP (Cocucci *et al.*, 2014). Synaptojanin-1, along with Hsc70 and auxilin, then disassemble (uncoat) the clathrin coat from the nascent vesicle by PI(4,5)P₂ to induce dissociation of clathrin adaptor proteins (Milosevic, 2018). Newly formed vesicles either fuse with endosomes-like intermediates or act as fusion-competent SVs as mentioned above. The sequence of events and the sequential recruitment of the various proteins involved in CME at synapses are summarised in figure 8.

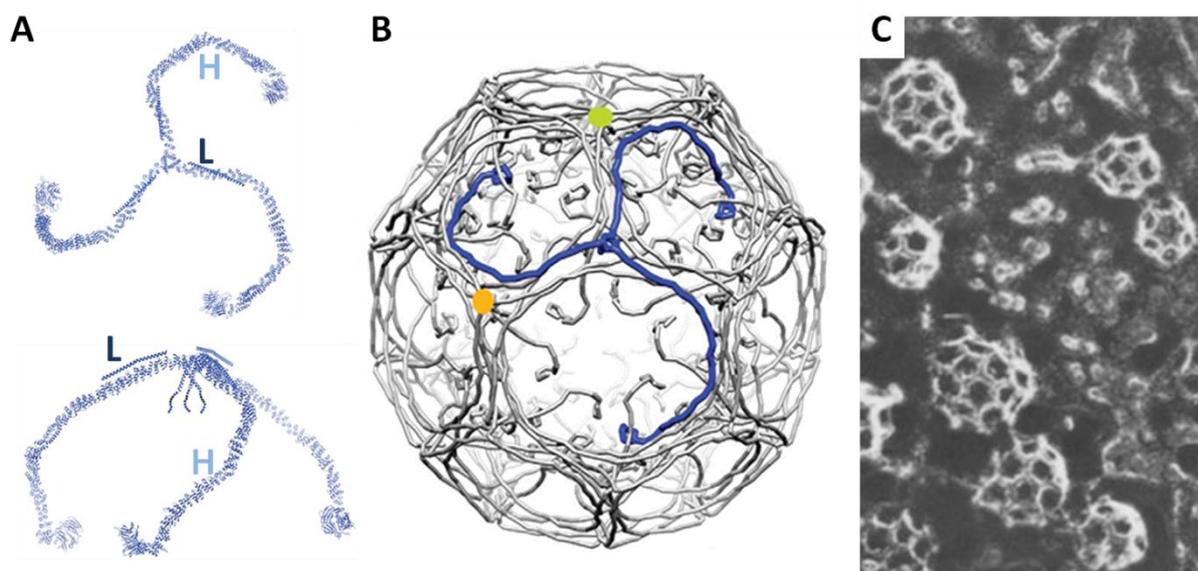


Figure 7: Clathrin triskelia and coat

A) The diagram depicts the clathrin triskelion viewed from different angles. H for heavy chain and L for light chain. **B)** Model of clathrin assembly that forms the clathrin coat. **C)** Basket-like structures that represent clathrin-coated vesicles observed in frog neuromuscular junction by freeze-fracture electron microscopy. **A)** and **B)** are adapted from (Xing *et al.*, 2010), and **C)** from (Heuser, 1980).

CME at synapses has been observed at various synapses over the last four decades and the significance of this mode of SV endocytosis has been evidenced through studies that interfere with the components of CME. For example, clathrin knockdown or dominant-negative inhibition of AP180 leads to a near-complete blockage of endocytosis at hippocampal synapses suggesting that CME accounts for the majority of membrane reuptake (Granseth *et al.*, 2006). In addition, knockdown of AP2 (Kim and Ryan, 2009) or knockout of auxilin (Yim *et al.*, 2010) resulted in significant impairment of CME at hippocampal synapses. Other studies in support of the presence and significance of CME at synapses are well described in a number of reviews (Saheki and De Camilli, 2012; L.-G. Wu *et al.*, 2014; Milosevic, 2018).

The recent controversies that surround CME at hippocampal synapses should also be discussed, as these observations seem to refute what has been well-accepted for the last four decades. It was discerned that most studies that put CME forward as an important mode of SV endocytosis were performed at room temperature (Watanabe *et al.*, 2014; Soykan *et al.*, 2017). Watanabe *et al.*, 2014, found that at physiological temperature, actin-dependent CIE is the dominant mode of endocytosis, taking up bulks of membrane from which clathrin and its adaptor proteins regenerate SVs (Watanabe *et al.*, 2014). Reducing the temperature significantly impairs actin polymerisation, and thus blocks the actin-dependent ultrafast endocytosis (a form of CIE; see 'CIE: ultrafast endocytosis' later) at synapses. There was an increase in the number of clathrin-coated pits on the presynaptic membrane, indicating a shift from ultrafast endocytosis to CME when the experiment is performed at room temperature. This suggested that CME is only dominant at room temperature, and that CIE is the dominant mode of endocytosis at physiological temperature. The dispensable nature of CME at synapses was also suggested in a study by Kononenko *et al.*, 2014, which shows that reducing the levels of clathrin or AP2 has very little impact on SV endocytosis during mild frequency stimulation and has no impact during high frequency stimulation (Kononenko *et al.*, 2014). This suggests that SV endocytosis occurs only partially as CME and dominantly as CIE at a range of stimulation frequencies. The study also indicates that the CIE involved retrieves membrane through a dynamin and endophilin-dependent mechanism to form endosomes-like structures, from which a clathrin-dependent mechanism regenerates SVs. More recently Soykan *et al.*, 2017, demonstrated that ultrafast endocytosis is likely to

be the dominant mode of endocytosis in response to a few initial action potentials, however, longer stimulations will favour a slower mode of endocytosis that is generally independent of clathrin and AP2 (Soykan *et al.*, 2017). The study goes on to explain that the CIE observed is heavily dependent on actin dynamics through formins, regulators of linear actin filaments. Such studies indicate a model in which CIE is the dominant mode of endocytosis under all stimulation conditions, forming endosomes-like structures, from which, clathrin-dependent reformation of SVs occur. It should be mentioned that the two studies discussed were performed in hippocampal neurons which has relevance to this thesis work.

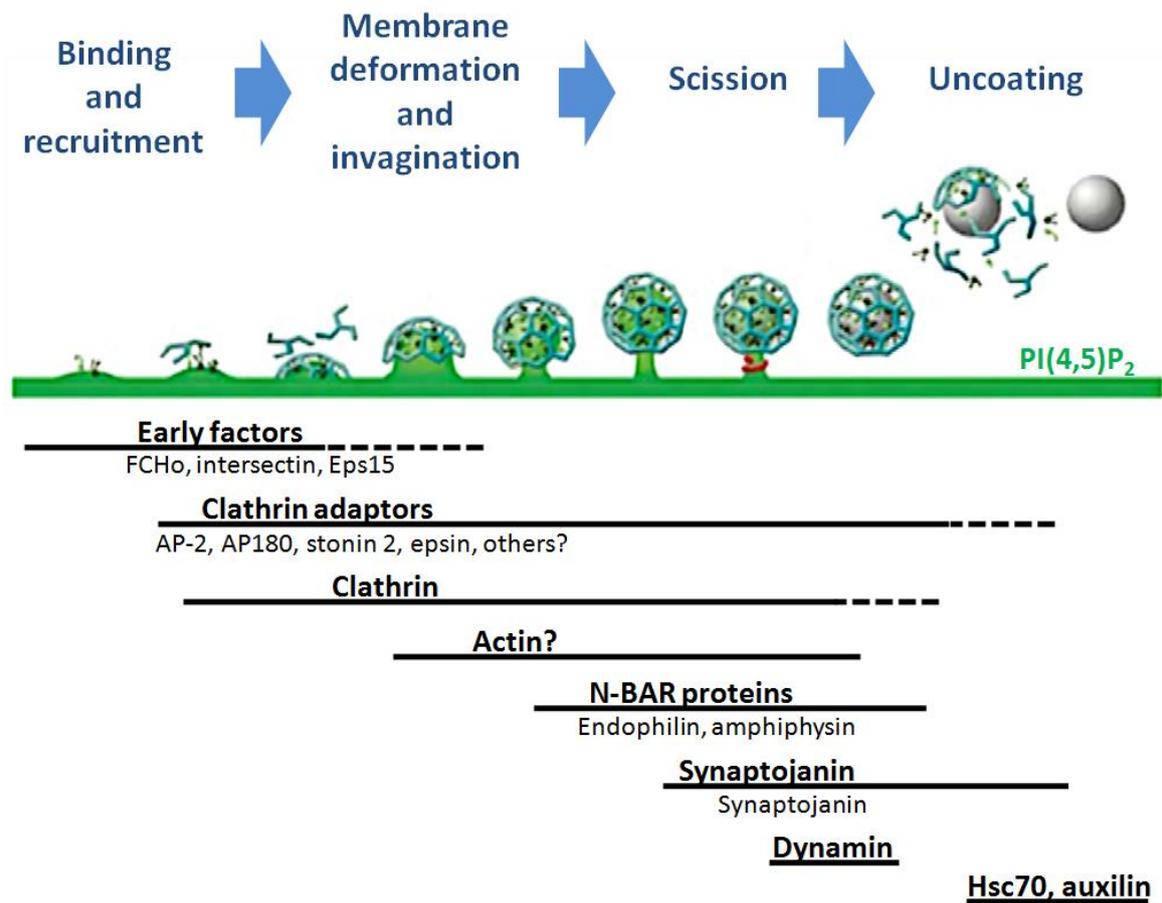


Figure 8: Clathrin-mediated endocytosis

The steps involved in clathrin-mediated endocytosis are given at the top and the sequential recruitment of the molecular actors involved is given at the bottom of the diagram. Clathrin-mediated endocytosis begins with ligand-receptor interaction (cargo of clathrin-mediated endocytosis which recruits FCHo (nucleator of clathrin-mediated endocytosis; Henne *et al.*, 2010). FCHo in turn recruits the structural proteins intersectin and Eps15. The recruitment of clathrin adaptors such as AP2 then follows. AP2 acts as a hub of interaction between the various molecular actors of clathrin-mediated endocytosis. Adapted from (Saheki and De Camilli, 2012).

1.3.4.2. Clathrin-Independent Endocytosis

A few forms of CIE occur in synapses which include the activity-dependent bulk endocytosis (ADBE) and ultrafast endocytosis (Milosevic, 2018). They differ in the size of membrane taken up, the rate at which they occur and the molecular actors that mediate them.

1.3.4.2.1. CIE: Ultrafast Endocytosis

This mode of endocytosis had only recently been described, and thus our knowledge is limited. Two recent studies used optogenetics in conjunction with ‘flash-and-freeze’ electron microscopy to demonstrate the existence of ultrafast endocytosis in two different model systems (mouse hippocampal synapses and *Caenorhabditis elegans* neuromuscular junctions) (Watanabe *et al.*, 2013b; Watanabe *et al.*, 2013a). This method involves light stimulation to induce a single or a pair of action potentials that cause neurotransmitter release. Then by the use of high-pressure freezer, cells are frozen at various defined time points before being observed under an electron microscope. These studies report that one action potential is enough to induce the fusion of one or two SVs around 30ms after stimulation. Such fusion events were quickly followed by compensatory endocytosis which occurred 50 to 100ms after stimulation, at sites immediately adjacent to active zones. Invaginations arising from such events generally lacked the classic coat, indicating that they are not clathrin-dependent, and the surface areas of these invaginations were estimated to be equivalent to four SVs. The studies also show that calcium influx alone is not enough to trigger ultrafast endocytosis, and that ultrafast endocytosis does not occur without exocytosis. Furthermore, it was suggested that actin dynamics is necessary to initialise membrane deformation, and dynamin function necessary for vesicle fission. SV regeneration then occurs from endosomes in a clathrin-dependent manner (Watanabe *et al.*, 2014). Finally the fact that ultrafast endocytosis occurs at a distinct site (within around 200nm of fusion sites) suggests a special membrane organisation in this region. More recently, it was demonstrated that endophilin-A and synaptojanin-1 are involved in ultrafast endocytosis by using synaptojanin-1 knockout or endophilin-A1/2 double knockout mice (Watanabe *et al.*, 2018). The study showed that the 5-phosphatase of synaptojanin-1 is required for the neck

formation in ultrafast endocytosis. Finally the fact that ultrafast endocytosis occurs at a distinct site (within around 200nm of fusion sites) suggests a special membrane organisation in this region (Watanabe *et al.*, 2013b; Watanabe *et al.*, 2013a).

1.3.4.2.2. CIE: Activity-Dependent Bulk Endocytosis

ADBE takes over as the main form of endocytosis when level of synaptic activity increases, when an exhaustive amount of fusion outbalances the rate of CME (Clayton and Cousin, 2009b; Saheki and De Camilli, 2012). First identified in frog neuromuscular junctions (Miller and Heuser, 1984), it is characterised by a large uptake of the presynaptic membrane to form endosomes-like structures called bulk endosomes in response to intense stimulation. The molecular aspect of ADBE is still under intense scrutiny, but recent works have shed light on some key molecules that may be involved in ADBE. Hypothetical molecular underpinnings of ADBE are depicted in figure 9.

A study by Wu *et al.*, 2009, suggests that calmodulin activation is necessary for ADBE (Wu *et al.*, 2009). Calcineurin is a calcium and calmodulin-binding phosphatase that has been implicated in ADBE. A study using an antagonist of calcineurin (cyclosporin A) suggested that calcineurin is crucial in allowing SV regeneration through ADBE (Cheung and Cousin, 2013). Calcineurin, when it binds calcium, dephosphorylates dynamin 1 to allow the interaction between dynamin 1 and syndapin 1, and this interaction is reported to be important in ADBE (Clayton *et al.*, 2009b). The role of dynamin 1 in ADBE, however, was challenged by a dynamin 1/3 double knockout study that indicated that ADBE proceeds unaffected (Y. Wu *et al.*, 2014). This study, nonetheless, provides a possible explanation for such a discrepancy by suggesting that dynamin 2, which is still present in very low levels, may replace the role of dynamin 1. More evidence in support of the role of dynamin 1 in ADBE exists. In fact, an earlier evidence for the involvement of dynamin 1 in ADBE was provided by a study on *Drosophila shibire* mutants (Van Der Blik and Meyerowitz, 1991). The multiple forms of dynamin encoded by the *shibire* gene in these mutants are rendered temperature-sensitive causing them to be inactive at low temperatures (Chen *et al.*, 1991; Van Der Blik and Meyerowitz, 1991). At low non-permissive temperatures, it was reported that tubular

invaginations remained attached to the plasma membrane, suggesting that the activity of dynamin is required for the scission of these invaginations to form endosomes-like structures (Van Der Blik and Meyerowitz, 1991). Moreover, it was shown that rephosphorylation of dynamin 1 is crucial to maintain ADBE in rodent neurons, as preventing rephosphorylation of dynamin 1 by cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 (GSK3) blocked the continuation of ADBE (Evans and Cousin, 2007; Clayton *et al.*, 2010). Finally, it was demonstrated that disrupting the interaction between dynamin 1 and syndapin 1 leads to the blockage of ADBE (Clayton *et al.*, 2009b). Taken together, it appears that dynamin is a key component of ADBE although the presence of the different isoforms may allow for functional redundancy.

The role of syndapin 1 in ADBE is unclear, but its molecular interactions provide hints. Syndapin 1 has binding sites for both N-WASP and dynamin (Qualmann *et al.*, 1999). N-WASP has a proline-rich domain (PRD), allowing its interaction with Src homology 3 (SH3) domains of various proteins, and it is able to interact also with actin and actin-related protein 2/3 (Arp2/3) complex via another region (Takenawa and Miki, 2001). In migrating cells, the recruitment of Arp2/3 allows for fast changes in the actin cytoskeleton, allowing the formation of filopodia. Such studies led to the hypothesis that syndapin could be the bridge between the actin cytoskeleton and dynamin in ADBE (Clayton and Cousin, 2009b).

The molecular underpinnings of ADBE was initially inferred through studies on a similar process such as macropinocytosis (Royle and Lagnado, 2003). Suppressing the dynamics of actin and the activity of phosphatidylinositol-3-kinase (PI3K) led to the impairment of macropinocytosis in non-neuronal cells, indicating the involvement of actin dynamics in this process (Nichols and Lippincott-Schwartz, 2001; Royle and Lagnado, 2003). It was then postulated that a similar mechanism may underlie ADBE in synapses. Evidence in support of this were then provided using frog neuromuscular junctions (Richards, Rizzoli and Betz, 2004) and retinal bipolar cells (Holt *et al.*, 2003), demonstrating that actin dynamics and PI3K activity are crucial for ADBE.

The possible connection between ADBE and PI3K implies that membrane lipids and lipid metabolism play a role in ADBE. Phosphatidylinositol phosphate kinase type I γ (PIP1 γ), an enzyme that synthesises PI(4,5)P₂, has been shown to be dephosphorylated by calcineurin under stimulation conditions that would induce ADBE (Sang *et al.*, 2005). The study

demonstrates that the dephosphorylated form of PIPK1 γ interacts with talin, which leads to the activation of PIPK1 γ and to increase in the level of PI(4,5)P₂ at synapses. This could mean a possible role of PI(4,5)P₂ in ADBE at synapses. Further speculations regarding the role of membrane lipids can be found in a review on ADBE by Clayton and Cousin, 2009 (Clayton and Cousin, 2009b). All in all, much of the molecular mechanisms underlying ADBE has been inferred from other studies and thus requires further investigation that will provide deeper insight.

It is already known that certain cargoes are taken up via CIE in non-neuronal cells. For example, β -integrins, glycosylphosphatidylinositol (GPI)-anchored proteins, interleukin-2 receptor alpha chain and major histocompatibility complex class I (MHCI) are all ingested via the ARF6-mediated endocytosis (Maldonado-Báez, Williamson and Donaldson, 2013). It seems quite plausible to postulate that a similar phenomenon may be true for CIE at synapses. A recent study on mouse cerebellar and hippocampal neurons showed that VAMP4 is a specific cargo of the ADBE (Nicholson-Fish *et al.*, 2015). In fact, the knockdown of VAMP4 leads to the blockage of ADBE suggesting that VAMP4 is not simply a cargo, but is a crucial element of the process. Interestingly, inhibiting ADBE does not affect the uptake of various SV proteins including synaptophysin and synaptotagmin-1. Clathrin heavy chain knockdown and pharmacological inhibition of CME led to reduced uptake of these SV proteins at high frequency stimulations suggesting that these SV proteins are taken up via CME. In contrast, an earlier study suggested that synaptotagmin-1 uptake during similar stimulation condition was unaffected by the impairment of CME using shRNA against clathrin heavy chains or pharmacological inhibition (Kononenko *et al.*, 2014). With the current understanding of SV recycling, it is difficult to explain the contradiction. A recent proteomic study revealed the ADBE core proteome which included cell adhesion and cytoskeletal molecules as well as trafficking proteins (Kokotos *et al.*, 2018). The study looks particularly into the Rab proteins that were purified with bulk endosomes and discover that the overexpression of Rab11 increases the number of synapses capable of ADBE and increases the rate of CME. Adaptor proteins AP1 and AP3, which were previously shown to be necessary for the regeneration of SVs from bulk endosomes (Cheung and Cousin, 2012), were also among the proteins purified with bulk endosomes (Kokotos *et al.*, 2018).

It is possible that ultrafast endocytosis and ADBE share similar molecular mechanisms, but the two modes of endocytosis differ in a few ways (Watanabe and Boucrot, 2017). Firstly, ultrafast endocytosis appears to occur closer to the active zone than ADBE and on a much faster time scale. Membrane internalisation via ultrafast endocytosis terminates in 100ms whereas ADBE takes several seconds to minutes (Richards *et al.*, 2000; Wu and Wu, 2007). Secondly, the amount of membrane internalised is more homogenous in ultrafast endocytosis, between 60 to 80nm in diameter, while no such property was observed with ADBE. Finally, the membrane taken up via ultrafast endocytosis will fuse with endosomes before protein sorting occurs, whereas the membranes taken up via ADBE, bulk endosomes, are suggested to be sorting stations.

Clathrin-independent endocytic modes at synapses seem to provide a way for restoring membrane tension at varying conditions; very rapid response to a single action potential or a series of action potentials in the case of ultrafast endocytosis; and large volumes of membrane during high frequency stimulation in the case of ADBE. Other than counteracting changes in membrane surface area, it seems possible that CIE at synapses retrieves SV proteins that are not retrieved by CME (possibly through cargo selection as in the example of VAMP4). A high-resolution time-lapse imaging study allowed showing that newly exocytosed SV proteins are clustered by interaction with the endocytic machinery, thus limiting movement by diffusion and restraining the clusters to the presynapse (Gimber *et al.*, 2015). Such pre-clustered SVs could then be taken up in large bulks of membrane during CIE before being placed in new single SVs through a clathrin and AP2-dependent mechanism (Kononenko *et al.*, 2014) or through another mechanism that is clathrin and dynamin-independent (Y. Wu *et al.*, 2014).

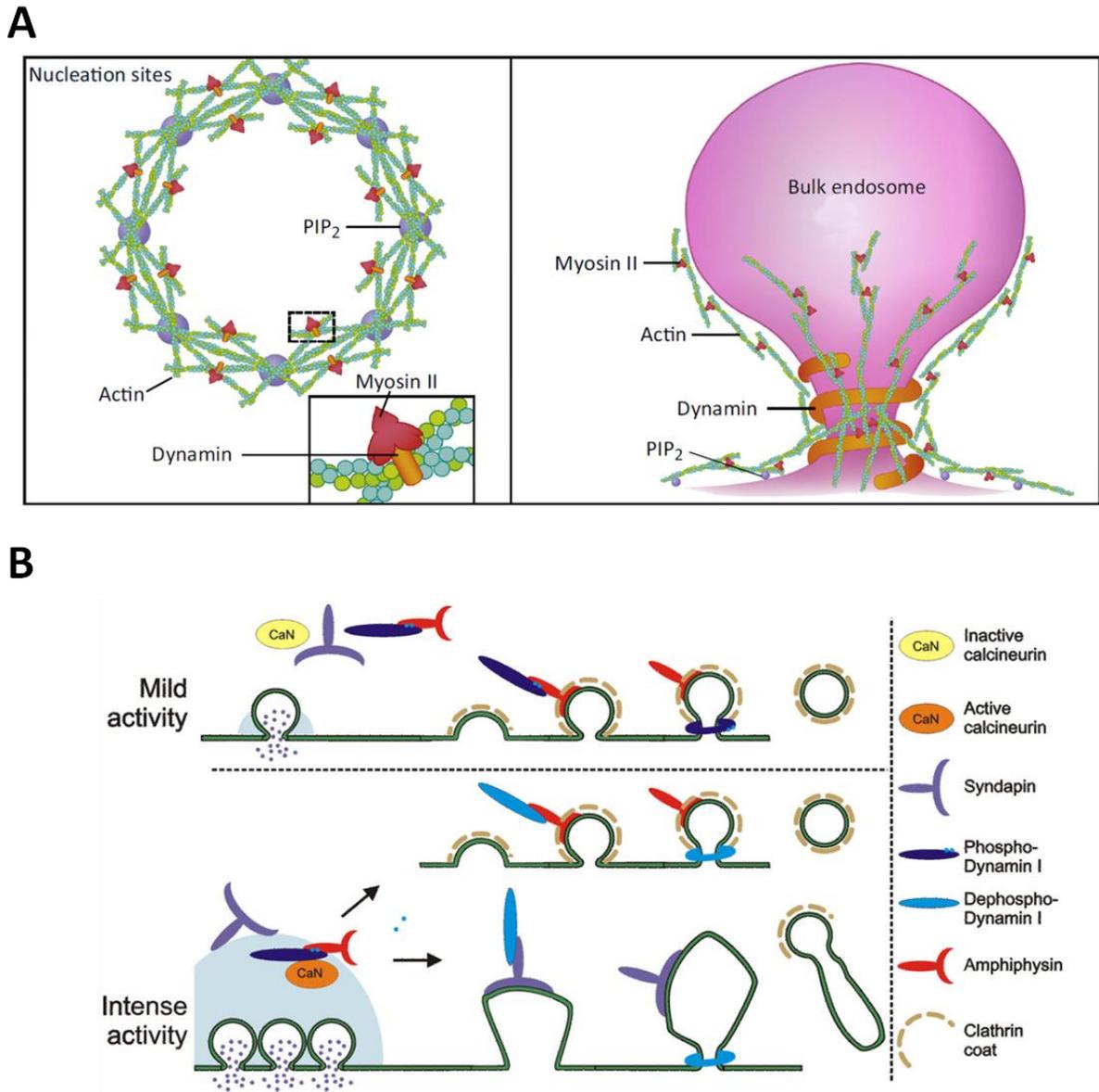


Figure 9: Activity-dependent bulk endocytosis

A) A possible molecular model of bulk endocytosis in chromaffin cells. PIP₂ refers to phosphatidylinositol 4,5-bisphosphate. PIP₂ mediated the initiation of actomyosin II rings that form around nascent bulk endosome. Dynamin and myosin are crucial to the formation of such constricting rings. Together, the actomyosin II ring and dynamin contribute to the fission process. Adapted from (Meunier and Gutiérrez, 2016). **B)** Dynamin 1 dephosphorylation is the key to the selection of SV endocytosis mode. Calcium microdomains are limited to the active zone during mild activity and thus calcineurin remains inactive. Intense activity leads to increased calcium microdomains that stretch beyond the active and activate calcineurin. Activated calcineurin in turn dephosphorylates dynamin 1 which allows the dynamin 1-syndapin interaction, allowing dynamin 1 to participate in bulk endocytosis. Taken from (Clayton and Cousin, 2009b).

1.3.4.3. Common Molecular Actors in Synaptic Vesicle Recycling

Beside the molecular actors involved in specific modes of endocytosis at synapses, there are certain actors, such as calcium, that are common to the different forms of endocytosis. As mentioned above, the arrival of action potential at the presynaptic terminal causes depolarisation and the consequent opening of voltage-gated calcium channels. During synaptic activity, the opening and closing of these channels are rapid and allow for high local increase in intracellular calcium (Kaeser and Regehr, 2014). It was demonstrated that calcium influx is one of the triggers of endocytosis at synapses (Wu *et al.*, 2009). However, the effect calcium influx has on SV endocytosis remains somewhat controversial. Numerous studies showed that reducing calcium influx by adding intracellular calcium chelators or reducing extracellular calcium slows down endocytosis at synapses (Ceccarelli and Hurlbut, 1980; Cousin and Robinson, 1998; Marks and McMahon, 1998; Moser and Beutner, 2000; Neves *et al.*, 2001; Sankaranarayanan and Ryan, 2001; Wu *et al.*, 2005). In agreement, other studies have shown that increased calcium concentration could speed up different endocytic modes in synapses of the central nervous system (Hosoi *et al.*, 2009; Wu *et al.*, 2009; Sun *et al.*, 2010). On the contrary, prolonged stimulation, and thus increased intracellular calcium was shown to slow down endocytosis in these synapses (Von Gersdorff and Matthews, 1994; Wu and Betz, 1996; Sankaranarayanan and Ryan, 2001; Sun, Wu and Wu, 2002; Balaji *et al.*, 2008). A recent study attempted to resolve this conflict and suggested that different stimulation conditions induce different patterns of intracellular calcium increase leading to varying effects on SV endocytosis (Wu and Wu, 2014). The study was based on dialysis of free calcium to vary the global intracellular calcium levels and different intensities of electrical stimulation. The study suggested that, in the calyx of held, global increase in intracellular calcium brought about by dialysis inhibits CME during mild stimulation conditions, but had no effect on any form of endocytosis during intense stimulation conditions. A number of studies on cultured hippocampal neurons expressing VGLUT-pHluorin also seem to suggest that calcium may underlie the stimulation-dependent changes in SV endocytosis which they observe (Armbruster *et al.*, 2013; Leitz and Kavalali, 2011; Li *et al.*, 2017). Leitz and Kavalali in 2011 demonstrated that increasing extracellular calcium concentration not only slows down single SV endocytosis, but also delays the onset of it (Leitz and Kavalali, 2011). With a similar experimental setup, an ensuing study revealed

that such an effect on SV endocytosis is not observed in synaptotagmin-1 knockdown neurons (Li *et al.*, 2017). It was further demonstrated that the interaction of synaptotagmin-1 with calcium and the membrane underlie its capacity to modulate the rate of SV endocytosis. Armbruster and his colleagues examined SV endocytosis in response to larger electrical stimulations at physiological temperatures (Armbruster *et al.*, 2013). It was reported that SV endocytosis accelerates during the initial 1 to 25 action potentials delivered at 10Hz, but slows in response to higher number of action potentials. The dephosphorylation of dynamin 1 and the consequent interaction of the dephosphorylated form with syndapin were implicated in the initial acceleration of SV endocytosis. Further experiments are needed to clarify and reconcile all the different observations, but these studies agree that calcium is crucial for SV endocytosis and that calcium influx is one of the triggers.

Calmodulin has been brought forward as an important calcium-sensor for endocytosis. A study carried out by Wu *et al.*, 2009, concludes that calcium influx is crucial for all forms of endocytosis and points to calmodulin as a possible calcium-sensor for SV endocytosis, since pharmacological blockage of calmodulin led to a partial impairment of SV endocytosis (Wu *et al.*, 2009). A function of calmodulin may be to regulate proteins that have the BAR-domain to augment their membrane-deforming properties (Myers *et al.*, 2016). Alternatively, calmodulin could have a role in SV pool regulation. In the calyx of Held, myosin light chain kinase, the activation of which is dependent on calmodulin, has been shown to regulate neurotransmission by modulating the readily releasable pool size (Srinivasan *et al.*, 2008). Calmodulin could also have a role through its interaction with calcineurin. Calcineurin was also implicated in all forms of SV endocytosis (Yamashita *et al.*, 2010; Wu *et al.*, 2014). It was suggested that calmodulin and calcineurin are involved in SV endocytosis in immature (postnatal day 7~8) rat calyces, by using inhibitors (Yamashita *et al.*, 2010). Under the same condition, SV endocytosis was unaffected in mature calyces (postnatal day 13~14) indicating a development-dependent change in calcium-sensor for SV endocytosis. A subsequent study by another group used CaNA α knockout (knockout of the α isoform of catalytic A subunit of calcineurin) and pharmacological inhibitors to find that while the knockout inhibited SV endocytosis in all developmental stages, the inhibitors produced similar results as the earlier mentioned study (Wu *et al.*, 2014). The discrepancy was explained by the fact that maturity leads to tighter coupling of calcium channels and the endocytic machinery, rendering the

inhibitor less effective in mature synapses. This suggested that calcineurin is essential for all forms of endocytosis at all developmental stages. Calcineurin, once activated, dephosphorylates its downstream effectors called dephosphins (Cousin and Robinson, 2001). Such dephosphins include AP180, dynamin 1, epsin, PIPKly and synaptojanin.

Finally, actin is also thought to be involved in all forms of SV endocytosis at synapses. Actin is highly abundant in presynaptic boutons and takes part in a number of different processes as reviewed by Rust and Maritzen, 2015, and summarised in figure 10. A recent study found that calyces and hippocampi from mice with either β -actin or γ -actin knockout resulted in significant impairment of various forms of endocytosis including slow endocytosis, fast endocytosis and ADBE (Wu *et al.*, 2016). The endocytic impairment was reversed when the expression of either isoform of actin was restored by transfection, but not when a polymerisation-deficient mutant form of actin was expressed. This indicated that actin polymerisation is a common requirement of the various forms of endocytosis. In addition, there are studies that show that pharmacological disruptions of actin dynamics lead to the impairment of specific modes of endocytosis at synapses. For example, more clathrin-coated pits were observed when lamprey synapses were treated with inhibitors of actin polymerisation, latrunculin B or swinholide A, or with inhibitors of actin depolymerisation, phalloidin, suggesting a role of actin dynamics in CME at synapses (Shupliakov *et al.*, 2002; Bourne, Morgan and Pieribone, 2006). In hippocampal neurons, it was shown that ultrafast endocytosis was completely blocked by latrunculin A treatment, indicating that actin polymerisation is crucial for ultrafast endocytosis (Watanabe *et al.*, 2013a; Watanabe *et al.*, 2013b). Taken together, these studies imply that actin dynamics is a common element shared by all forms of endocytosis at synapses.

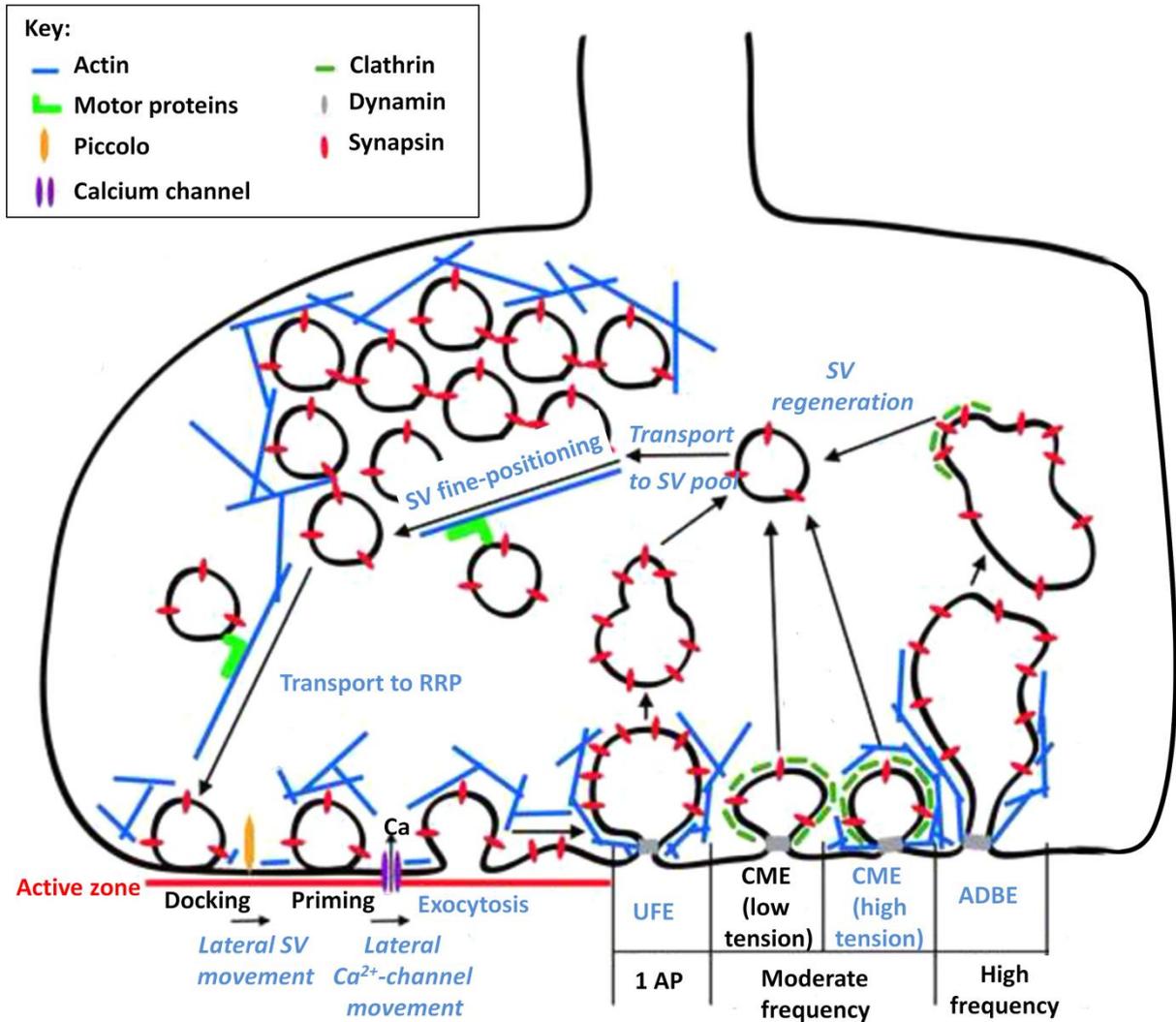


Figure 10: A summary of the function of actin in a presynaptic bouton

The processes in which actin is known to act are written in blue and normal font and processes in which actin is thought to act are in blue and italics. Black font is used for processes that do not involve actin. Abbreviations: AP, action potential; UFE, ultrafast endocytosis; RRP, readily releasable pool. Adapted from (Rust and Maritzen, 2015).

1.3.5. Exo-Endocytosis Coupling

Neurotransmission involves exocytosis for the release of neurotransmitters and compensatory endocytosis to regenerate the expended SV and accumulating evidence suggest that exocytosis and endocytosis at synapses are linked or coupled.

1.3.5.1. Cytomatrix of the Active Zone

It has been suggested that various constituents of CAZ may help couple exocytosis and endocytosis. The components of CAZ have been proposed to regulate the localisation of calcium channels since the downregulation of various CAZ proteins led to reductions in the number of calcium channels in the active zone, reduced calcium currents and reduced SV exocytosis (Gundelfinger and Fejtova, 2012). Not only are CAZs important in coupling calcium influx to exocytosis, they may also be regulators of SV endocytosis. Piccolo, for instance, has been found to interact with the SH3 domain of auxin binding protein 1 (Abp1) through its proline-rich region (PRR) (Fenster *et al.*, 2003). Adp1 in turn interacts with actin and dynamin 1, which lead to speculations that piccolo could indirectly regulate SV endocytosis. In support of this idea, blocking the interaction between Adp1 and dynamin 1 has been shown to negatively regulate CME (Kessels *et al.*, 2001). Additionally, a study on *Drosophila* demonstrated that G protein coupled receptor kinase 2 interacting protein (GIT), a component of CAZ which interacts with piccolo, localises to the periphery of active zones and associates with stoned B (stonin 2 in mice) (Podufall *et al.*, 2014). Loss-of-function mutation of GIT resulted in partial mislocalisation of stoned B and impairment of CME, indicating that GIT regulates SV endocytosis.

1.3.5.2. Exocytosis and Synaptic Vesicle Proteins

In most cases of synaptic transmission, the amount of membrane fused via exocytosis equates the amount of membrane retrieved by endocytosis. In fact, it has been suggested that exocytosis is a trigger of endocytosis. It was shown that SV endocytosis is significantly impaired in neurons that are treated with neurotoxins that cleave SNARE proteins (such as

SNAP25) to effectively block exocytosis (Hosoi *et al.*, 2009; Yamashita *et al.*, 2011; Xu *et al.*, 2013). It was suggested that this is due to the involvement of these SNARE proteins in SV endocytosis. This was further supported by a study on cultured hippocampal neurons in which SNAP25 or synaptobrevin expression was reduced by knockdown (Zhang *et al.*, 2013). SV endocytosis was significantly impaired in such cultures, supporting the notion that SNAREs are involved in SV endocytosis. Other studies allowed demonstrating that certain SNARE proteins are involved in the initiation of SV endocytosis. A number of interactions between SNARE proteins and endocytic proteins support such a view. For example, there are interactions between SNAP25 and intersectin (Okamoto, Schoch and Südhof, 1999), and syntaxin and dynamin (Galas *et al.*, 2000). More direct evidence of the involvement of SV proteins in endocytosis have also been put forward. Synaptotagmin-1, as mentioned earlier, is the calcium sensor for SV fusion. Yao and colleagues used hippocampal neurons from synaptotagmin-1 knockout mice and various mutant forms of synaptotagmin-1 to show that this protein plays a differential role in exocytosis and endocytosis (Yao *et al.*, 2012). To overcome the tight coupling between exocytosis and endocytosis, they used different mutants that helped demonstrate that the changes seen in SV endocytosis in the different experimental groups was not due to changes in exocytosis, effectively dissecting out the two differential roles of synaptotagmin-1. Taken together, it would seem that certain SV proteins added to the presynaptic membrane upon SV fusion will associate with endocytic proteins to promote SV endocytosis.

1.3.5.3. Membrane Lipids

Other plausible candidates of exo-endocytosis coupling include the membrane lipids that are involved in both exocytosis and endocytosis. A number of interactions between negatively charged lipids (such as phosphatidylinositol phosphates or PIPs) and SV priming proteins have been reported (Lauwers, Goodchild and Verstreken, 2016). It was suggested that motifs with positive charges (such as the C2 domain) govern such interactions. Synaptotagmin-1 is a SV protein which contains a C2 domain and it has recently been implicated in SV priming which was heavily dependent on the level of PI(4,5)P₂ (Walter *et al.*, 2017). The study found that increasing the level of PI(4,5)P₂ enhances SV priming and

thereby increases the size of readily releasable pool. This enhancement was abolished in synaptotagmin-1 or Munc13-2 knockout chromaffin cells. A mechanism by which membrane lipids augment exocytosis may be through shielding of electrostatic repulsion between SV proteins. In β -cells of the pancreas, it was shown that PIPs and phosphatidylserines facilitate the interaction between VAMP2 and syntaxin-1A by reducing electrostatic repulsion as the two proteins bind for vesicle exocytosis (Williams *et al.*, 2009; Lauwers *et al.*, 2016).

Further evidence supporting the role of PI(4,5)P₂ in exocytosis have been provided. Super-resolution STED (stimulated emission depletion) microscopy helped reveal microdomains of PI(4,5)P₂ clusters (of around 73nm) in PC12 cells (model cells of neurosecretion), that recruited syntaxin-1A by electrostatic interaction (Van Den Bogaart *et al.*, 2011). A study on chromaffin cells reports that reducing levels of PI(4,5)P₂ by overexpression of a membrane-targeted version synaptojanin's phosphatase domain leads to weakened exocytosis (Milosevic *et al.*, 2005). On the other hand, increasing levels of PI(4,5)P₂ by infusion through patch pipetting or overexpression of PIPK1 γ leads to augmented exocytosis. In addition to PI(4,5)P₂, it was suggested that phosphatidylinositol 3,4,5-trisphosphate PI(3,4,5)P₃ also has a role in SV exocytosis. A study on *Drosophila* found PI(3,4,5)P₃ to mediate such clustering effect on syntaxin-1A, and that reducing the level of PI(3,4,5)P₃ leads to defects in neurotransmission (Khuong *et al.*, 2013). Similar outcomes were observed in cortical neurons of mice (Paolo *et al.*, 2004). In PIPK1 γ knockout neurons, some synaptic transmission defects were observed that strongly suggested defective SV endocytosis and possible problems with SV exocytosis.

Membrane lipids are also important in SV endocytosis. It was mentioned earlier that PI(4,5)P₂ may have a role in ADBE, but it has also been suggested that PI(4,5)P₂ may also play a role in other modes of endocytosis. It was shown that knocking out PIPK1 γ in mice led to decreased levels of PI(4,5)P₂ and a concomitant defect in SV endocytosis in neurons (Paolo *et al.*, 2004). This suggested that PI(4,5)P₂ is important in SV endocytosis. The study also suggested possible defect in SV exocytosis suggesting that PI(4,5)P₂ might be one of the links between SV exocytosis and endocytosis. Varying the level of PI(4,5)P₂ has later been suggested to influence CME at various stages (Antonescu *et al.*, 2011), further asserting the importance of membrane lipids in endocytosis. Besides the studies discussed in this chapter, a considerable amount of evidence pointing to the importance of membrane lipids and their

metabolism in SV recycling have recently reviewed (Maritzen and Haucke, 2017; Walter *et al.*, 2017).

1.4. Synaptic Plasticity

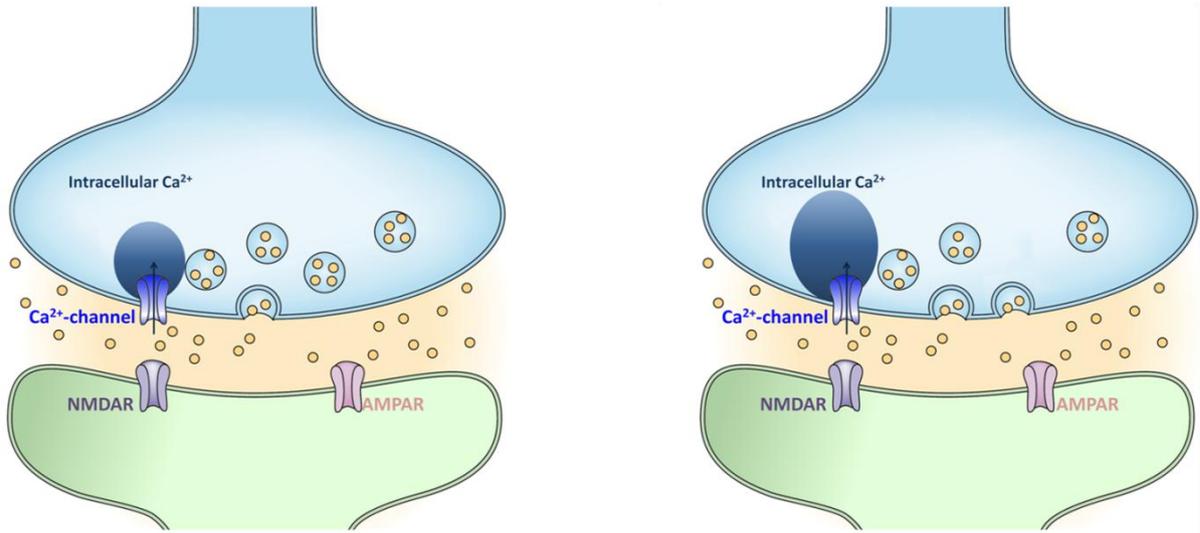
Synapses go through changes over time in response to changes in synaptic activity that either strengthens or weakens them (Citri and Malenka, 2008; Ho, Lee and Martin, 2011). This is referred to as synaptic plasticity and it is currently accepted that synaptic plasticity is at the centre of learning and memory. Electrophysiological studies have allowed the observation of such changes occurring at synapses and depending on the duration, can be classified into short-term plasticity and long-term plasticity.

1.4.1. Short-Term Plasticity

Synaptic changes grouped under short-term plasticity last between milliseconds to minutes (Citri and Malenka, 2008). Such phenomena are considered vital to the short-lived active memory, and rapid adaptations to sensory inputs. A few forms of short-term plasticity exist and several molecular mechanisms have been proposed to govern these.

Short-term plasticity manifest as depression (decrease) or facilitation (increase) of postsynaptic response to either two stimuli in close intervals or tetanic stimulation (Citri and Malenka, 2008; Regehr, 2012). The former can be measured by two stimuli with short time intervals, the first acting as the conditioning stimulus and the second reporting a change in synaptic strength. The ratio between the two is referred to as the paired-pulse ratio (PPR) with the increase being referred to as paired-pulse facilitation (PPF) and decrease paired-pulse depression (PPD). Such changes last between hundreds of milliseconds to several seconds. Finally, post-tetanic depression or post-tetanic potentiation occurs in response to trains of stimulation and the changes can last up to several minutes.

A



B

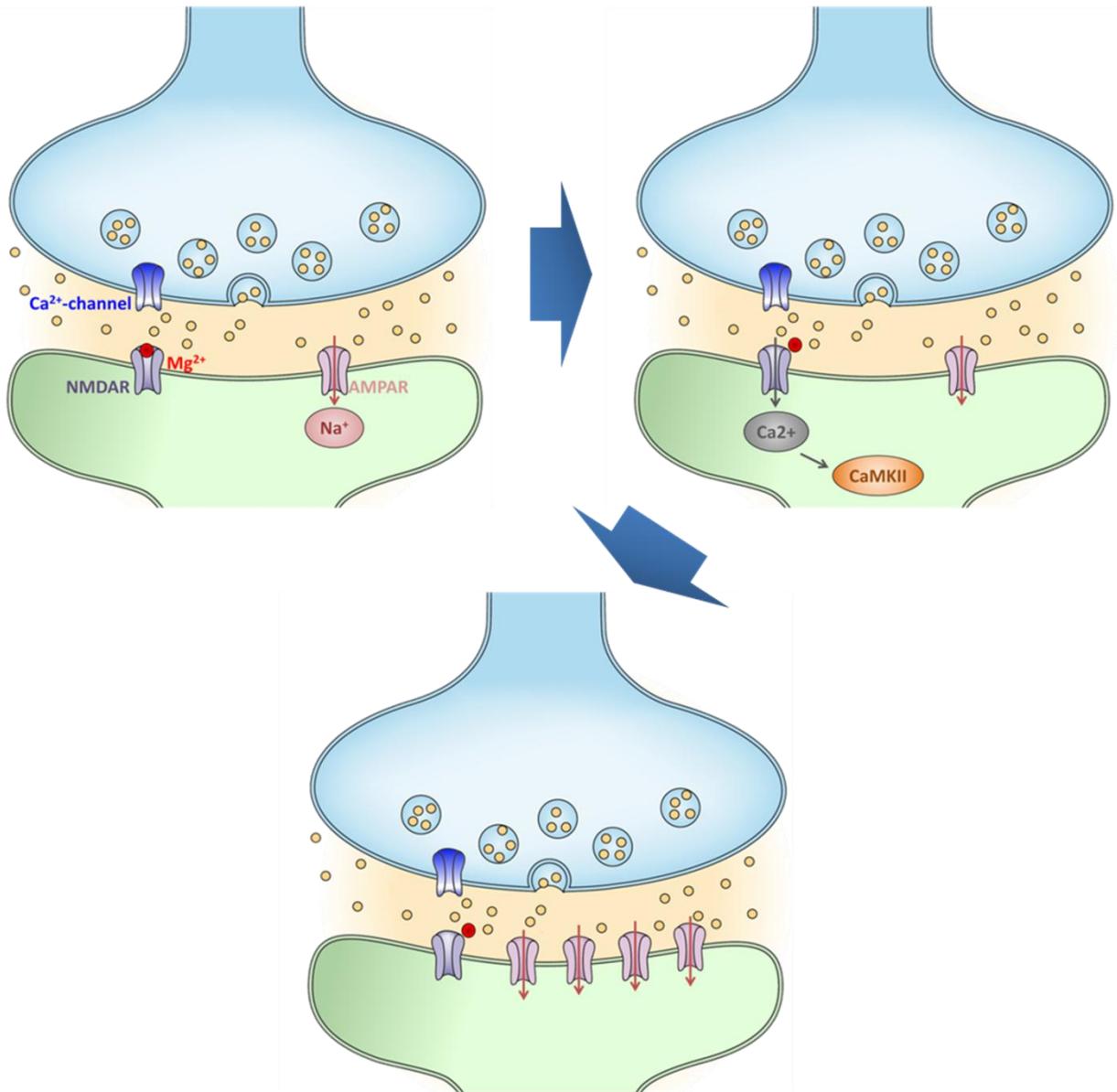


Figure 11: Synaptic plasticity (↑)

A) Paired-pulse facilitation brought about by residual calcium. More intracellular calcium is available for synaptic vesicle fusion during the second stimulation (right panel) compared to the first stimulation (left panel), leading to increased synaptic vesicle fusion. Abbreviations: AMPAR, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; NMDAR, N-Methyl-D-aspartic acid receptor. **B)** Long-term potentiation leading to increased AMPAR on the postsynaptic terminal. Depolarisation caused by the opening of AMPAR and glutamate binding to NMDAR lead to the displacement of Mg^{2+} from the NMDAR, and the subsequent influx of Ca^{2+} . This in turn activates CaMKII for the increase in synaptic AMPAR. **A)** and **B)** are adapted from (Nicoll and Schmitz, 2005)

The presynaptic elements that give rise to short-term plasticity include regulation of intracellular calcium and properties of SVs (Citri and Malenka, 2008). In the case of PPD or PPF, the first stimulus will cause the opening of voltage-gated calcium channels allowing a rapid rise in local calcium concentration around the channels. Synaptotagmins detect such rises in calcium to allow fusion of SVs. Once the channels close, the residual calcium dissipates by diffusion and binds other proteins. A study found that synaptic depression was caused by calmodulin-mediated inactivation of calcium channels leading to reduced calcium influx (Mochida *et al.*, 2008). In terms of PPF, the residual calcium (calcium remaining after the first stimulation) may allow the release of more SVs in response to the second stimulus giving rise to PPF (see figure 11; Citri and Malenka, 2008). Regulation of intracellular calcium in nerve terminals underlies PPF, and it is thought that endogenous calcium-binding proteins alter PPF by regulating intracellular calcium levels. Calbindin is a calcium-binding protein with high on-rate which has been found to be strongly expressed in the mouse hippocampus (Blatow *et al.*, 2003). Calbindin knockout led to reduced PPF, and this effect was reversed when nerve terminals were loaded with BAPTA or calbindin, indicating a role for calbindin in regulating PPF. It was argued that fast calcium-binding proteins, such as calbindin, bind calcium that enters synaptic terminals during the first stimulation. Due to saturation, less calcium is buffered during the second stimulation allowing the release of more neurotransmitters.

Another factor that regulates short-term plasticity is the release probability. Release probability refers to the propensity of SVs to release their content, and this could differ between synapses and individual SVs (Körber and Künner, 2016). In paired-pulse experiments

the synapses with high release probability exhibits depression in response to the second stimulus while synapses with low release probability exhibit facilitation (Citri and Malenka, 2008; Regehr, 2012).

A number of causes for post-tetanic depression and post-tetanic potentiation have been put forward. Post-tetanic depression is thought to occur when the recycling pool has been depleted at a synapse by a prolonged stimulation and as the stimulation is also high-frequency, replenishment does not compensate for the depletion (Regehr, 2012). Other causes of post-tetanic depression include the inhibition of exocytosis triggered by signalling cascade or desensitisation of postsynaptic receptors (Citri and Malenka, 2008). In the case of post-tetanic potentiation, it has been suggested that gradual accumulation of residual calcium resulting from trains of stimuli could saturate calcium buffers at synapses and thus lead to synaptic facilitation (Katz and Miledi, 1968; Rosenthal, 1969; Weinreich, 1971; Citri and Malenka, 2008). Another theory suggests that a shift of calcium sensor from synaptotagmin to one with a higher affinity would allow increase in the release probability, leading to facilitation (Citri and Malenka, 2008). SV endocytosis has an effect on post-tetanic potentiation as demonstrated in a study on dynamin-1 knockout mice (Mahapatra and Lou, 2017). Dynamin-1 knockout, which was previously reported to bring about an impairment of CME (Ferguson *et al.*, 2007), led to enhanced post-tetanic potentiation probably arising from increase SV size (Mahapatra and Lou, 2017).

1.4.2. Long-Term Plasticity

Long-term plasticity refers to synaptic modifications that are long-lasting and are thought to contribute to learning and memory, and (Citri and Malenka, 2008). Long-term synaptic facilitation is called long-term potentiation (LTP) and depression is called long-term depression (LTD).

LTP is mostly studied in mammalian hippocampus as this part of the brain is thought to be important for learning and memory storing. LTP is a phenomenon observed in electrophysiological studies where a conditioning train of high-frequency stimulation leads to long-lasting enhanced postsynaptic depolarisation, or increased amplitude of excitatory

postsynaptic potential (EPSP)⁶, in response to the subsequent stimulations (Bliss and Lømo, 1973). One of the earliest work on LTP and memory demonstrated that the induction of N-Methyl-D-aspartic acid receptor (NMDAR)-mediated LTP in rat hippocampi was necessary for spatial memory (Morris *et al.*, 1986). Since then many studies showed the involvement of NMDARs in the induction and maintenance of LTP (see Citri and Malenka, 2008; and Ho *et al.*, 2011). During basal synaptic activity, postsynaptic depolarisation caused by sodium influx is mostly mediated by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA), while NMDARs contribute little due to the blockage of its channel by extracellular magnesium. When the magnesium ions are removed and the channels open, NMDARs allow the entry of sodium and calcium. Depolarisation of the postsynaptic membrane and glutamate binding triggers the dissociation of the magnesium ion and thereby unblock NMDARs. High frequency stimulations induce the opening of NMDARs leading to the entry of calcium, a secondary messenger which triggers a signalling cascade for the induction and maintenance of LTP. The indispensable nature of calcium (Lynch *et al.*, 1983) and calmodulin kinase II (CaMKII) (Silva *et al.*, 1992) have been well established, and a number of other kinases have been implicated (Citri and Malenka, 2008). Potentiation is brought about by either an increase in the conductivity of AMPARs mediated by CaMKII-dependent phosphorylation of the receptor (Derkach *et al.*, 1999) or the fusion of AMPAR-containing vesicles with the postsynaptic membrane to increase the number of AMPAR on the postsynaptic membrane (see figure 11; Park *et al.*, 2004; Kopec *et al.*, 2006). Additionally, it was shown that calcium-bound CaMKII also interacts with NR2B subunits of NMDARs rendering itself autonomous, or calcium-independent (Bayer *et al.*, 2001; Halt *et al.*, 2012).

Although much work on LTP has focussed on the postsynaptic mechanisms, there are presynaptic mechanisms that are thought to underlie LTP (summarised in figure 12). Presynaptic LTP is mostly observed in mossy fiber synapses of hippocampi. The different mechanisms proposed all lead to increased neurotransmitter release (Nicoll and Schmitz, 2005; Citri and Malenka, 2008). These include presynaptic kainate receptors that enhance neurotransmitter release in response to the huge amount of glutamate released by high frequency stimulation trains (Contractor *et al.*, 2001; Schmitz *et al.*, 2003) and the gradual

⁶ EPSP is a temporary depolarisation of the postsynaptic membrane potential caused by ions flowing through ion-channels that are activated by neurotransmitters.

accumulation of intracellular calcium leading to a signalling cascade that involves adenylyl cyclase (Villacres *et al.*, 1998), cyclic adenosine monophosphate (cAMP) (Weisskopf *et al.*, 1994) and protein kinase A (PKA) (López-García *et al.*, 1996). Additionally, SV pools such as the readily releasable pool is suggested to play an important role in synaptic plasticity. It has been demonstrated that LTD is accompanied by a reduction in the readily releasable pool size (Goda and Stevens, 1998), while LTP is accompanied by an increase in the readily releasable pool (Tyler *et al.*, 2006).

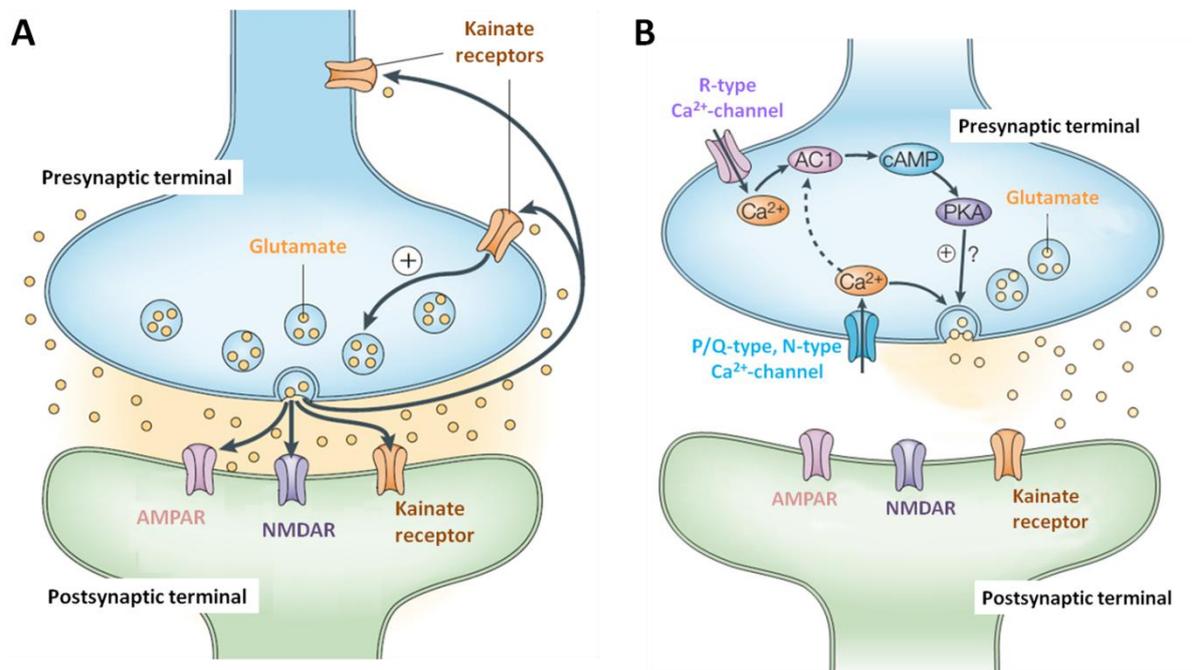


Figure 12: Presynaptic long-term potentiation

A) A model of long-term potentiation involving presynaptic kainate receptors. Glutamate from the synaptic cleft diffuses out to activate presynaptic kainate receptors. Abbreviations: AMPAR, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; NMDAR, N-Methyl-D-aspartic acid receptor. **B)** A model of long-term potentiation involving R-type calcium channels. Abbreviations: AC1, adenylyl cyclase 1; cAMP, cyclic AMP; PKA, protein kinase A. **A)** and **B)** are adapted from (Nicoll and Schmitz, 2005).

Altogether, the various processes at synapses and the mechanisms that underpin work in close conjunction with each other to maintain healthy synapses. A number of molecular actors show functional redundancies that ensure that synapses could function even in the absence of these molecular actors, while the absence of others could lead to the disorganisation and improper functioning of synapses.

2. The Protein Alix

ALG-2 interacting protein X (Alix), which is also known as apoptosis interacting protein 1 (AIP1) is a ubiquitous cytosolic protein first described through its calcium-dependent association with apoptosis linked gene 2 (ALG-2) (Missotten *et al.*, 1999; Vito *et al.*, 1999). Alix is highly conserved in eukaryotes showing widespread expression in different organisms ranging from yeasts and flies to mice and zebrafish. This allowed the study of Alix in various model systems, leading to the discovery of a range of cellular processes that Alix is involved in and the numerous interactions it shares (see figure 13). The underlying molecular mechanisms of Alix in such processes, however, are still poorly characterised. This chapter aims to provide a general overview of the protein Alix.

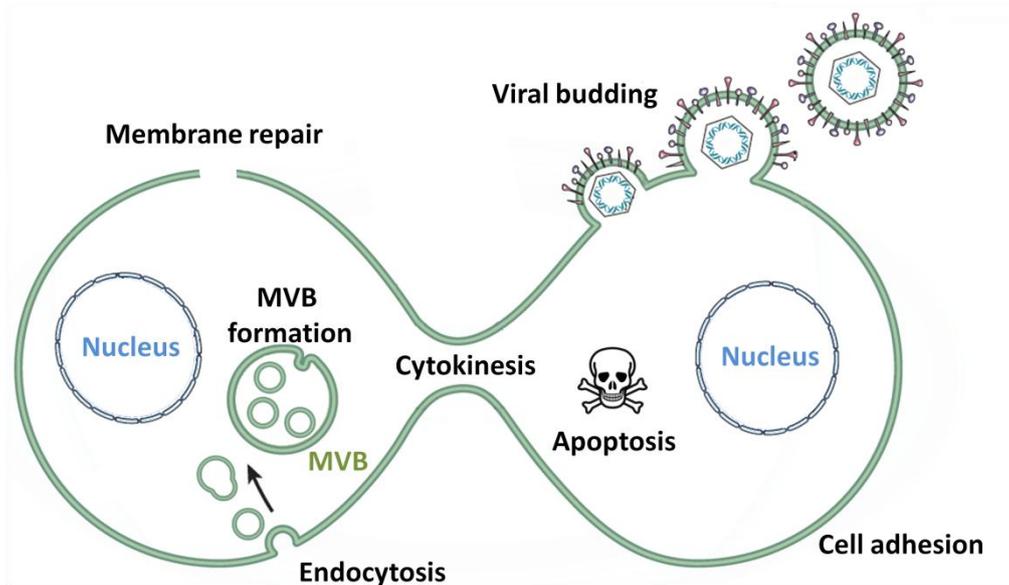


Figure 13: Cellular processes involving Alix

The diagram depicts a summary of the cellular processes that involve Alix. MVB stands for multivesicular body. Endocytosis and MVB formation encompasses the role of Alix in the endo-lysosomal pathway, intraluminal vesicle formation, exosomes biogenesis and backfusion. Adapted from (Raiborg and Stenmark, 2009)

2.1. Structure and Interactions

Alix is an 868 amino acid long protein and comprises three domains: the N-terminal Bro1 domain, the central V domain and the C-terminal proline rich domain (PRD); as summarised in figure 14.

The Bro1 domain consists of 358 amino acids at the N-terminal end and is made up of 3 helices in 'α-helical hairpin' (Fisher *et al.*, 2007). The amino acid residues Asp97 and Asp178 are crucial for the ability of Alix to bind calcium while Lys104 and Phe105 allow interactions with lysobisphosphatidic acid (LBPA) (Bissig *et al.*, 2013). Ile212 mediates the interaction with charged multivesicular body protein 4 (CHMP4) of the endosomal sorting complexes required for transport-III (ESCRT-III) complex (Katoh *et al.*, 2003; Fisher *et al.*, 2007). Tyr319 interacts with the SH2 domain of Src to allow the subsequent phosphorylation of Alix (Schmidt, Dikic and Bögler, 2005), and this phosphorylation differentially regulates the interaction of Alix with its partners (Dejournett *et al.*, 2007; Zhou *et al.*, 2009). Finally, the Bro1 domain also binds calcium with high affinity (EC50 <1μM; Bissig *et al.*, 2013).

The V domain, between amino acids 362 and 702, encompasses eleven α-helices that form two arms, which fold together allowing dimerisation of Alix in a head-to-tail formation (Strack *et al.*, 2003; Pires *et al.*, 2009). A motif in this domain allows recognition of and binding to the YPXL motif of GAG viral proteins of human immunodeficiency virus (HIV) and equine infectious anemia virus (EIAV) viruses (Strack *et al.*, 2003). Other interactors of this domain include protease activated receptor 1 (PAR1) (Dores *et al.*, 2012), syntenin (Baietti *et al.*, 2012) and ubiquitin (Dowlatshahi *et al.*, 2012).

The PRD, 166 amino acids at the C-terminal, is a region enriched in proline and tyrosine and contains numerous SH3 domain binding motifs. The SH3 domain proteins that interact with the PRD of Alix include ALG-2, centrosomal protein 55 (CEP55), Cbl-interacting protein of 85kDa (CIN85) (Chen *et al.*, 2000), endophilins-A1, -A2 and -A3 (Chatellard-Causse *et al.*, 2002), Nedd-4 ubiquitin ligase, and tumour susceptibility gene-101 (Tsg-101) (Strack *et al.*, 2003).

Additional interactions have also been identified such as galectin-3, lectin involved in CIE (Chen *et al.*, 2009). Interactions with some of the constituents of actin polymerisation through the Bro1 and V domains have also been suggested, in a study involving truncated forms of the two domains. These include actin, α-actinin and cortactin (Pan *et al.*, 2006).

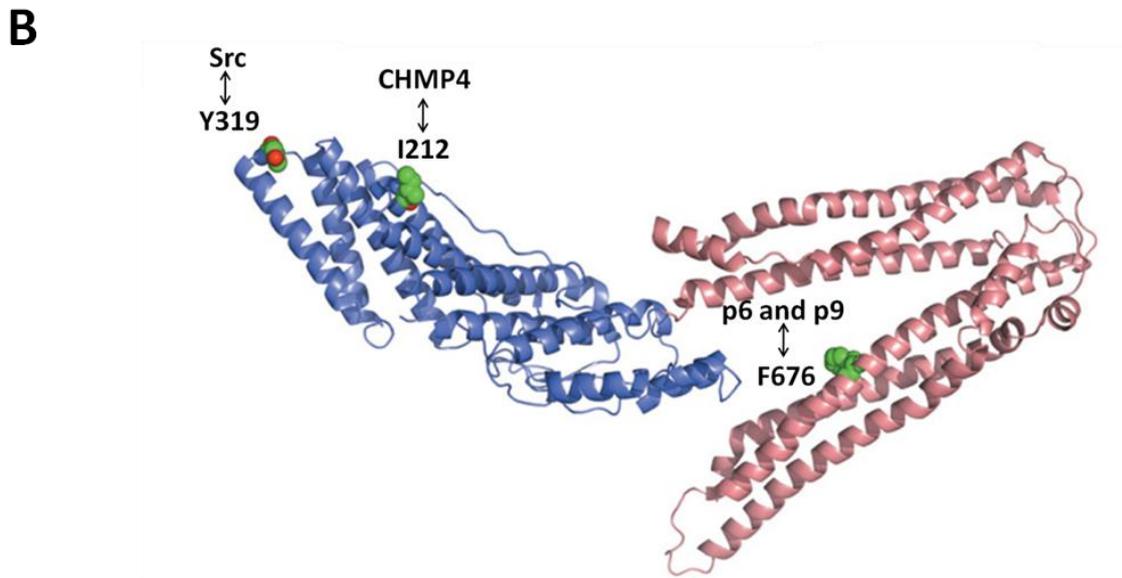
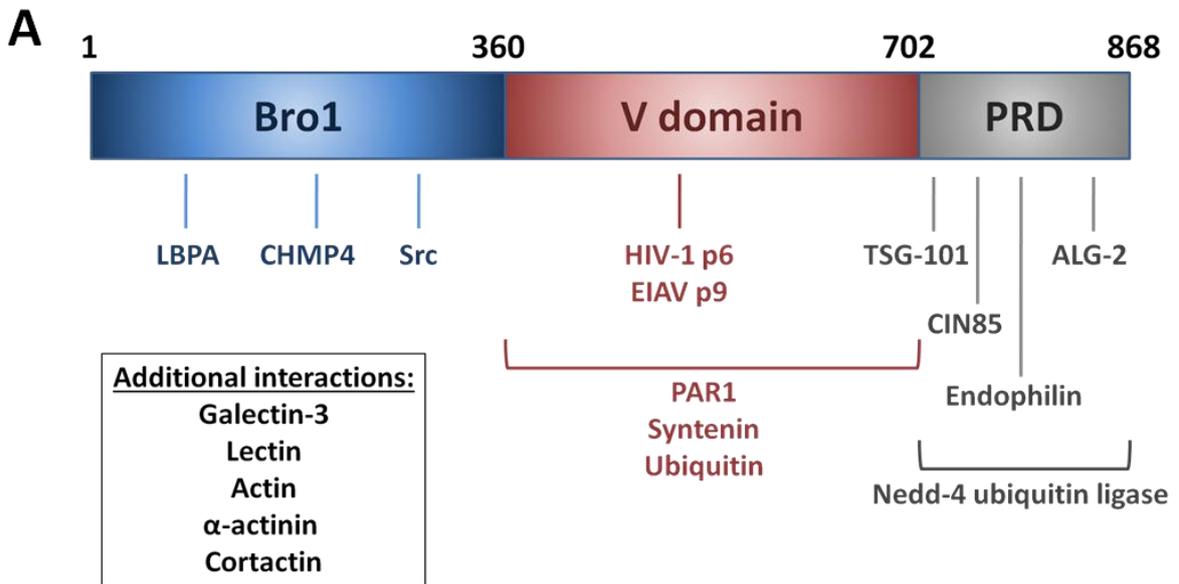


Figure 14: Structure of Alix

A) The three domain that comprise Alix (Bro1, V and proline-rich domains, PRD) and the interacting proteins. **B)** The structures of Bro1 (blue) and V domains (red) and 3 residues that are important for various interactions. **A)** and **B)** are adapted from (Fujii *et al.*, 2007).

2.2. Functions

2.2.1. ESCRT and Membrane Budding.

The role of Alix revolves around membrane deforming and trafficking, and in particular, it works in close association with the ESCRT machinery (see figure 15) that act to deform and bud off membranes. The complexes of the ESCRT and their accessory proteins that comprise the ESCRT machinery include the ESCRT-0, I, II, and III complexes, Alix and vacuolar protein sorting 4 (Vps4) (Schmidt and Teis, 2012; Sadoul *et al.*, 2017). In contrast to the endocytic machinery that pulls the membrane towards the cytosol, the ESCRT complexes act to push cellular membranes away from the cytosol. Generally ESCRT-mediated membrane budding occurs by sequential recruitment of various constituents of the ESCRT machinery to deform the membrane followed by the constriction of the neck from the inside (Barelli and Antony, 2009; Wollert *et al.*, 2009). Our current understanding is that the ESCRT-III is at the core of membrane deformation and neck constriction (see figure 16). It was demonstrated that the overexpression of CHMP2B alone induced 150µm-long protrusions at the plasma membrane (Belly *et al.*, 2010; Bodon *et al.*, 2011). Moreover, the CHMP4B of the ESCRT-III is able to polymerise into helical filaments that circularise and mediate the deformation of the membrane (see figure 16; Hanson *et al.*, 2008; Barelli and Antony, 2009; Bodon *et al.*, 2011). The scission of the budding membrane is only completed when the ATPase Vps4 is recruited to disassemble the ESCRT-III complex (Hanson *et al.*, 2008). These studies suggest a crucial role of the ESCRT-III and Vps4 in cellular processes that involve the deformation and budding of the membrane away from the cytoplasm.

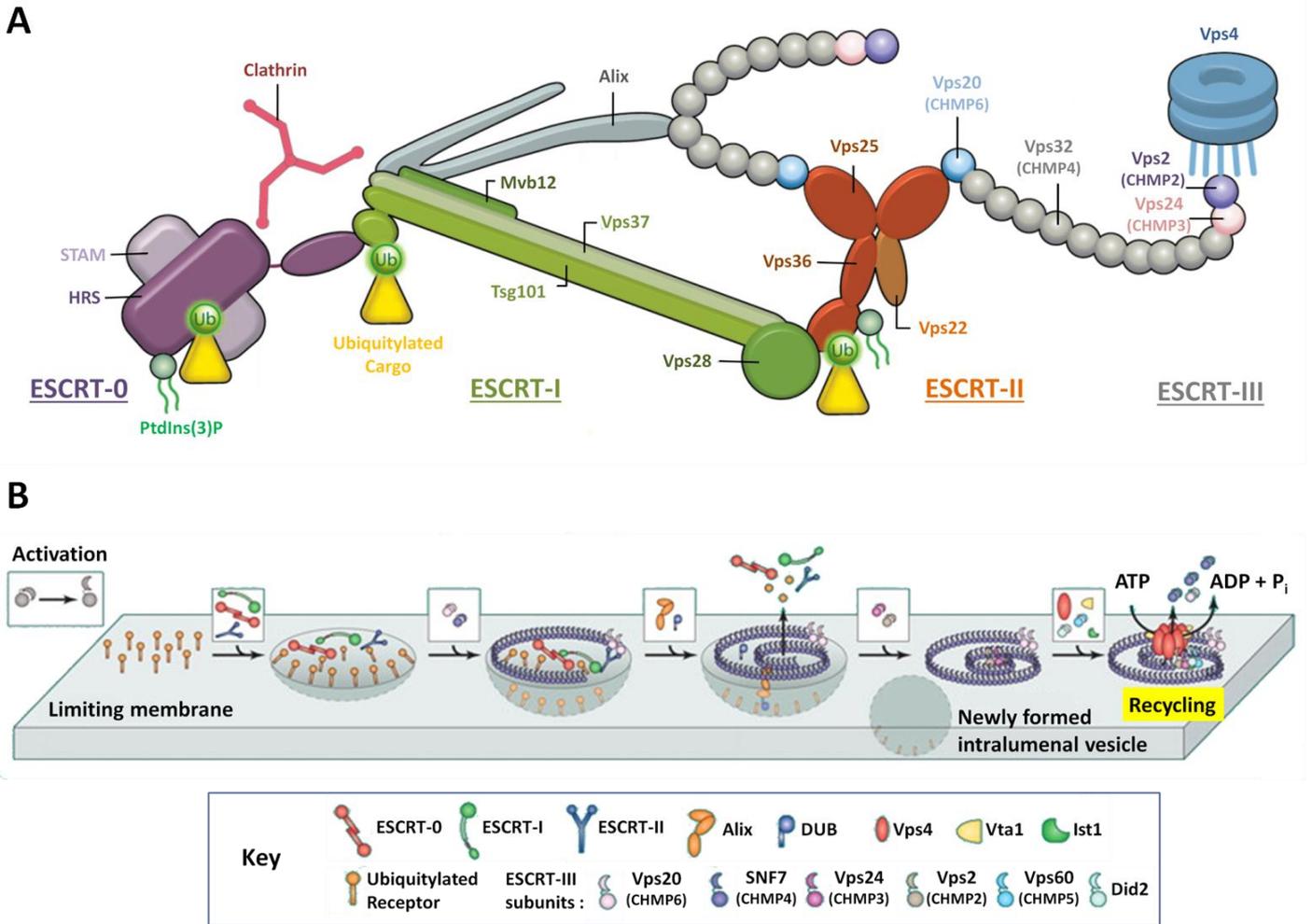


Figure 15: the ESCRT machinery

A) The diagram depicts the interactions between the four ESCRT complexes, accessory molecules and ubiquitinated cargo. Adapted from (Raiborg and Stenmark, 2009). **B)** The diagram depicts the role of the ESCRT machinery in multivesicular body formation. Adapted from (Wollert *et al.*, 2009).

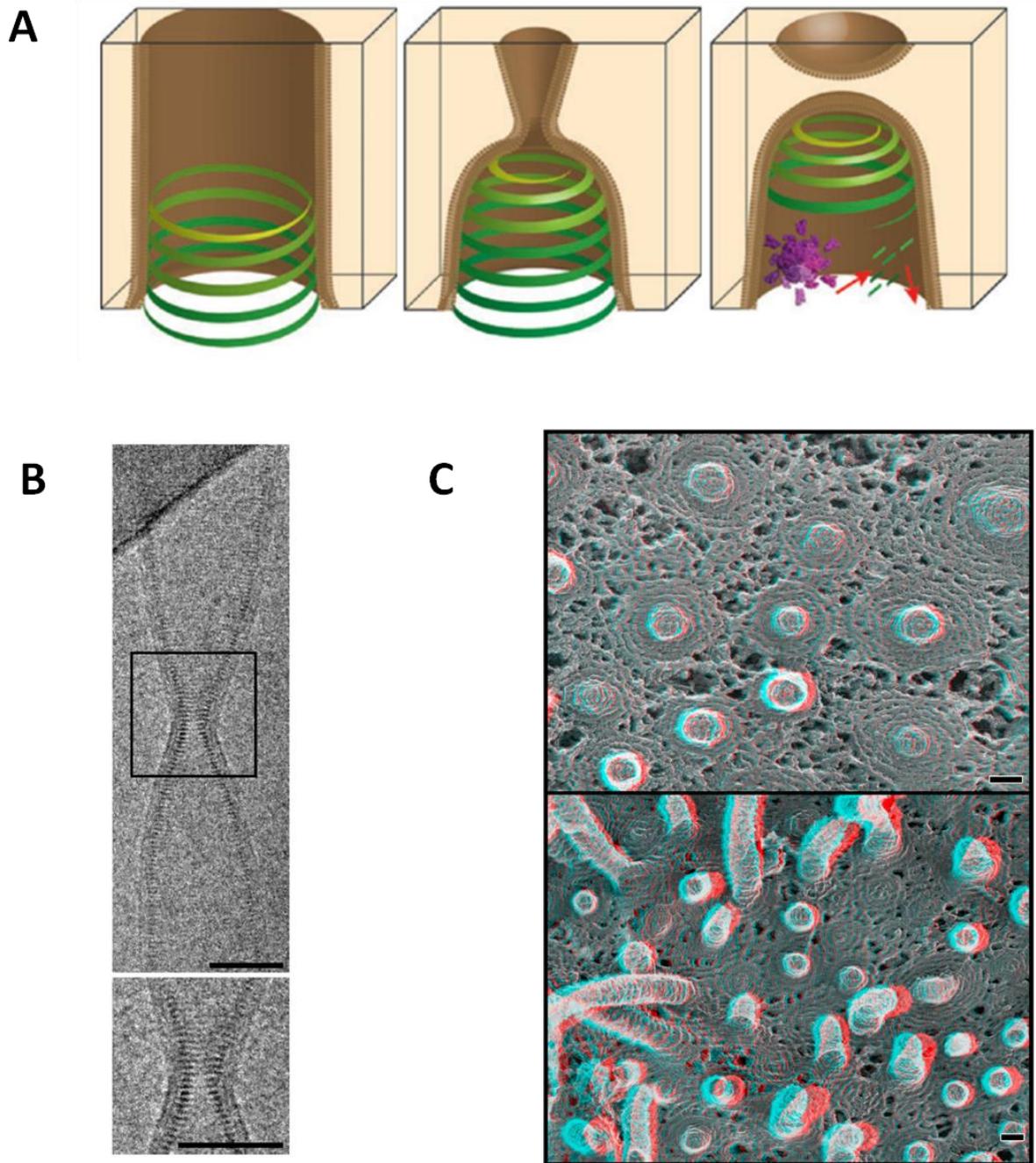


Figure 16: ESCRT and membrane deformation

A) a model of membrane deformation mediated by components of the ESCRT machinery such as CHMP4 that polymerise to form helical structure. Taken from (McCullough, Colf and Sundquist, 2013) **B)** Electron cryomicroscopy images showing CHMP2B tubes formed in HeLa cells. Taken from (Bodon *et al.*, 2011). **C)** Deep-etch electron microscopy images of CHMP4B polymers forming spirals on the plasma membrane to generate membrane tubules. Taken from (Hanson *et al.*, 2008).

2.2.2. Endosomal-Lysosomal Pathways

Endocytosis allows for the internalisation of cell surface receptors that have varying functions and mechanisms for the reception and execution of extracellular signals (Hu *et al.*, 2015). The Receptors, once ingested in endocytic vesicles, will be incorporated into the endosomal pathway by fusing with endosomes where sorting occurs either for recycling back to the plasma membrane or degradation via the lysosomal pathway. In the latter case endosomes mature to form multivesicular bodies (MVBs) that contain internal vesicles called intraluminal vesicles (ILVs). This occurs by the inward budding of the endosomal membrane carried out by the ESCRT machinery that prevents further signalling from the internalised receptors. The MVBs then fuse with lysosomes exposing the ILVs to the lumen of lysosomes allowing for the degradation of receptors by the action of lysosomal acid hydrolases. It is now well-accepted that the formation of some ILVs is heavily dependent on the ESCRT machinery and Alix appears to have a role in this process.

Cell surface receptors that are destined for degradation will be ubiquitinated. Members of the ESCRT machinery have ubiquitin binding domains. ESCRT-0 are first to bind to ubiquitinated receptors on endosomes leading to the sequestering of the receptors. Then sequential recruitment of the ESCRT-I and ESCRT-II follows that are mediated by interactions between various components of the complexes including HRS, Tsg-101, Vps28 and Vps36. The interaction between Vps25 of ESCRT-II and CHMP6 of ESCRT-III prepares the endosome for ESCRT-III assembly. Deubiquitination of the receptors then takes place via deubiquitinases that are recruited by the ESCRT-III complex (Schmidt and Teis, 2012). Finally, the ESCRT-III components polymerise and facilitate the budding of the endosomal membrane before their disassembly by Vps4 which leads to fission.

The involvement of Alix in this pathway was first suggested through experiments on yeast. The depletion of BRO-1, a homolog of Alix in yeast, leads to the accumulation of ubiquitinated receptors on endosomal membrane surfaces, implying that BRO-1 is crucial for the sorting of ubiquitinated receptors (Springael *et al.*, 2002). It was then shown that BRO-1 regulates the recruitment of Doa4 for the deubiquitination of receptors, a step which is crucial for the formation of MVBs (Luhtala and Odorizzi, 2004). However, studies on Alix in mammals indicated that receptor degradation can continue without the need for Alix. The

depletion of Alix in mammalian cells had very little effect on the degradation of EGFR (Cabezas *et al.*, 2005; Luyet *et al.*, 2008; Mercier *et al.*, 2016). Alix from mammals and BRO-1 from yeast are only 22% identical indicating a possible differential role and thus providing an explanation for these studies (Sadoul, 2006). Instead, His domain phosphotyrosine phosphatase (HDPTP), a homolog of Alix which also contains a Bro1 domain, was implicated in the degradation of EGFR (Doyotte *et al.*, 2008). A recent study refuted this notion and instead supported the role of Alix in receptor degradation. Sun *et al.*, 2015, found that downregulation of Alix or disruption of its interaction with CHMP4 or itself results in decreased degradation of EGFR through MVBs (Sun *et al.*, 2015). The authors suggest the existence of receptor degradation not involving MVBs and that this is the basis for the discrepancies with the previous findings that Alix is not essential for receptor degradation. In support, it was shown that varying the level of Alix affects ILV formation. LBPA are lipids that are enriched in MVBs and are vital to the formation of ILVs (Bissig *et al.*, 2013). Alix appeared to be able to regulate the presence of LBPA and cholesterol on liposomes (Matsuo *et al.*, 2004) and late-endosomes (Chevallier *et al.*, 2008) which highly influences the formation of ILVs in these membrane-bound structures. The interaction between Alix and LBPA may influence the number of ILVs in MVBs through another mechanism called backfusion (Bissig *et al.*, 2013). This is the process by which ILVs fuse back with the outer MVB membrane to release its content into the cytoplasm. Backfusion is hijacked by viruses such as the vesicular stomatitis virus (VSV) to release viral material into the cytoplasm in an Alix dependent manner (Le Blanc *et al.*, 2005; Bissig *et al.*, 2013).

2.2.3. Exosome Biogenesis

Alternatively, instead of fusing with lysosomes, MVBs can release their content by fusing with the plasma membrane. The ILVs, once released, are referred to as exosomes and they have been implicated as vessels used in intercellular communication (Chivet *et al.*, 2013). It was recently found that the interaction between Alix and syntenin allows the formation of a syndecan-syntenin-Alix complex that regulate exosome biogenesis (Baietti *et al.*, 2012). The study showed that the knockdown of Alix led to reduced exosomes containing CD-63 and syndecans. A study that followed demonstrated that the knockdown of Alix led to increase in

the number of large exosomes that contained the major histocompatibility complex II (MHC II) (Colombo *et al.*, 2013). The authors suggest that their findings do not contradict the earlier finding that depleting Alix reduces exosomes biogenesis, but instead show different mechanisms of exosomes biogenesis in different cell types, and that Alix may play a role in the sorting of cargoes into ILVs.

2.2.4. Endocytosis

The Alix-CIN85 interaction has been implicated in the regulation of epidermal growth factor receptor (EGFR) internalisation (see figure 17). Once its ligand binds, the EGFR becomes activated and in turn recruits and phosphorylates Casitas B-lineage lymphoma (Cbl) (Soubeyran *et al.*, 2002). Cbl then recruits CIN85 along with its partner endophilin, and this complex of receptors and its interacting proteins lead to the endocytosis of the EGFR. Evidence supporting the role of Alix in the endocytosis of EGFR comes from a study on HeLa cells which showed that reducing the level of Alix led to a slight increase in the endocytosis of EGFR (Schmidt *et al.*, 2004). On the other hand, the overexpression of Alix led to decreased endocytosis. It was suggested that Alix compete with Cbl for CIN85 and thus negatively regulates EGFR endocytosis by preventing the formation of the endocytic complex. It appears that Src is the regulator of this inhibitory role of Alix since Src phosphorylates Alix which blocks its interaction with CIN85 allowing the relocation of Alix to the cytosol (Schmidt, Dikic and Böglér, 2005). These experiments were performed at low concentrations of EGF which would trigger CME.

The use of Alix KO mouse embryonic fibroblasts (MEFs) allowed demonstrating that CIE is significantly reduced in the absence of Alix, while CME continues unaffected (Mercier *et al.*, 2016). For example, the uptake of cargoes of CIE such as cholera toxin chain B (CTxB), β 1 chain of integrin and β chain of interleukin-2 receptor were significantly reduced while the uptake of transferrin receptor remained the same in Alix KO MEFs. It was demonstrated that the interaction of Alix with CIN85 and endophilin are necessary to promote CIE to internalise cargo and regulate cell migration. The interaction between Alix, CIN85 and endophilin has already been studied in HeLa cells in studies mentioned above (Schmidt *et al.*,

2004; Schmidt *et al.*, 2005). The results of Mercier, *et al.*, 2016, showed that EGFR degradation at high concentration of EGF (which triggers CIE of the EGFR) was delayed in Alix KO MEFs compared to wild-type cells, while EGFR degradation was unaffected at low concentration of EGF which induces CME exclusively (Mercier *et al.*, 2016). This was supported by a preceding study which demonstrated that EGFR degradation, triggered by high concentration EGF, was delayed in HEK cells when Alix expression was reduced (Sun *et al.*, 2015). These results are in conflict with previous findings which show that reducing the level of Alix increases EGFR degradation (Schmidt *et al.*, 2004; Schmidt *et al.*, 2005) at low concentrations of EGF, or that there is no change (Cabezas *et al.*, 2005) at high concentrations of EGF. However, it should be noted that these studies were performed on HeLa cells, whereas Mercier *et al.*, 2016, was performed on fibroblasts. The discrepancy may simply come from the two different types of cells that harbour different molecular mechanisms to degrade EGFR.

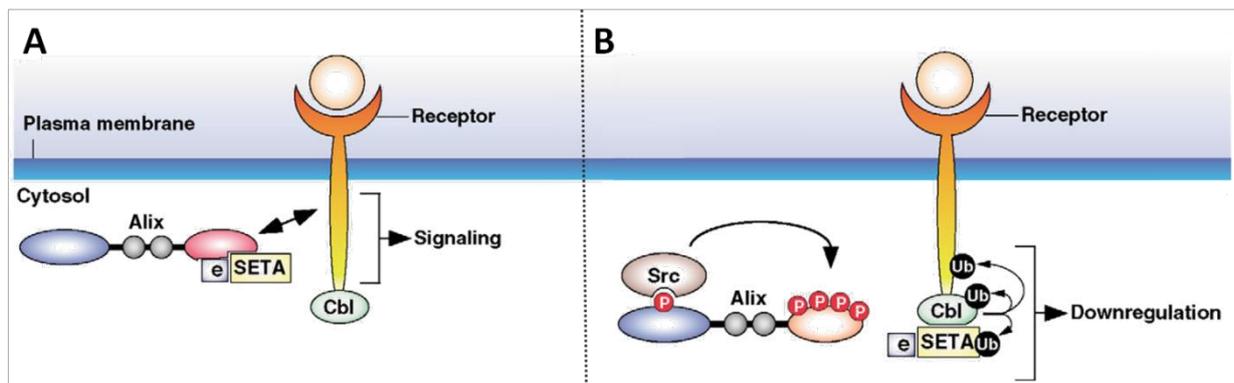


Figure 17: Alix and EGFR endocytosis

A) The non-phosphorylated form of Alix associates with the activated EGFR dimer (represented as monomer) and prevents the interaction between the endophilin-SETA complex with Cbl by binding endophilin and SETA. This prevents the ubiquitylation of EGFR and the subsequent endocytosis of the receptor. **B)** The phosphorylation of Alix. Abbreviations: e, endophilin; Ub, ubiquitin; SETA, SH3- domain-containing expressed in tumorigenic astrocytes.

2.2.5. Membrane Repair

It is well accepted that when the plasma membrane is damaged in eukaryotes it causes the influx of extracellular calcium, triggering a repair mechanism (Andrews *et al.*, 2014). Membrane repair occurs by one of three mechanisms: 1) excision of damaged section by endocytosis or budding (similar to viral budding), 2) closure of wound by constriction or 3) patching by the addition of lysosomes. Two recent studies implicated Alix and components of the ESCRT machinery in membrane repair (Jimenez *et al.*, 2014; Scheffer *et al.*, 2014). The influx of calcium upon damage to the membrane is the trigger of membrane repair and is detected by ALG-2 (Scheffer *et al.*, 2014). ALG-2 is a well-conserved 22-kDa protein which is composed of five EF-hand structures (Maki *et al.*, 2011). ALG-2 binds calcium at micromolar concentration and changes its conformation which allows interaction with its interacting proteins including Alix. It is a ubiquitous protein that localises to various membranous structures upon binding calcium, and in this case, it is recruited to the plasma membrane to allow the recruitment of Alix (Scheffer *et al.*, 2014). The recruitment of Alix is quickly followed by the recruitment of CHMP2A, CHMP3, CHMP4B, Tsg-101 and Vps4 allowing the excision of damaged membrane. Depletion of these proteins lead to reduced membrane repair, albeit to a varying degree (Jimenez *et al.*, 2014; Scheffer *et al.*, 2014). The underlying mechanism still remains elusive, but it seems conceivable that CHMP4B polymerisation allows the closure and expulsion of the damaged section of the membrane, a manner that resembles viral budding (Jimenez *et al.*, 2014).

2.2.6. Viral Budding

Viruses hijack the molecular machinery of the host cell to replicate their genetic material, produce proteins necessary for their assembly, and then leave the host cell once assembled. Retroviruses such as the HIV-1 and EIAV take advantage of the ESCRT machinery to leave the host cell by budding (Sundquist and Krausslich, 2012). The viral GAG proteins, regulators of virus assembly, recruit Alix via the YPXL motif (Strack *et al.*, 2003) and Tsg-101 via their YPXL and PTAP motifs (Garrus *et al.*, 2001) to the plasma membrane. Alix and Tsg-101 then recruit components of the ESCRT-III and Vps4 (Jouvenet *et al.*, 2011) for the budding of new viruses through a mechanism that resembles cytokinesis (Carlton *et al.*, 2008;

Sundquist and Krausslich, 2012).

2.2.7. Cell Adhesion

Cell adhesion is the way in which cells attach and interact with neighbouring cells or the extracellular matrix and it is in close association with other cellular processes like signal transduction and migration. A study by Wu *et al.*, 2002, suggested that Alix may have a role in cell adhesion (Wu *et al.*, 2002). The study was performed in HeLa cells and showed that the overexpression of HP95, a human ortholog of Alix, reduced tumourigenicity in nude mice and promoted anoikis in cultured cells. Anoikis is a form of programmed cell death which occurs in response to detachment from the extracellular matrix. The study does not provide the underlying mechanism, but does indicate a link between Alix and cell adhesion. Subsequent studies on focal adhesion then provided deeper insight into the molecular underpinnings. Focal adhesions are bridges that connect a cell with the extracellular matrix and the various constituents include actin regulatory proteins, integrins and different enzymes such as focal adhesion kinases (Wozniak *et al.*, 2004). Focal adhesion kinases are regulators of focal adhesions that are activated by phosphorylation. A study by Schmidt *et al.*, 2003, discovered that Alix, only once bound to CIN85, is able to inhibit cell adhesion by preventing activation of focal adhesion kinases (Schmidt *et al.*, 2003). An ensuing study showed that the phosphorylation of Alix by Src prevents its interaction with CIN85 and leads to increased cell adhesion (Schmidt, Dikic and Bögler, 2005). These studies suggest a regulatory role of Alix in cell adhesion. The finding that Alix is capable of interacting directly with components of the actin cytoskeleton (Pan *et al.*, 2006) further endorses a role of Alix in cell adhesion.

2.2.8. Cell Death

As mentioned, Alix was first identified as a molecular interactor of ALG-2, a protein implicated in calcium-dependent apoptosis. Apoptosis, or programmed cell death, is a process driven by cysteine proteases called caspases that allow the death of cells. One of the pioneering works suggested that Alix works in conjunction with ALG-2 in apoptosis since the

overexpression of a truncated form of Alix prevents apoptosis (Vito *et al.*, 1999). A subsequent study on astrocytes revealed that CIN85 is involved in UV-induced apoptosis (Chen *et al.*, 2000). Such a function of CIN85 was shown to be dependent on its interaction with Alix and ALG-2, indicating the involvement of Alix in apoptosis. A number of other studies also implicate Alix in apoptosis. It was demonstrated that an increase in the level of endogenous Alix preceded kainate-induced apoptosis in hippocampal neurons (Hemming *et al.*, 2004) and 3-nitropropionic acid-induced neurodegeneration in striatal neurons (Blum *et al.*, 2004). Additionally, it was demonstrated that increasing the level of Alix in cerebellar granule neurons (CGN) was enough to induce caspase-dependent cell death (Trioulier *et al.*, 2004). Furthermore, overexpressing the C-terminal part of Alix or a mutant form that does not bind ALG-2 prevent apoptosis.

Apoptosis can be triggered in two ways: the intrinsic mitochondrial pathway involving caspase-9 and the membrane-receptor induced extrinsic pathway involving caspase-8. The binding of tumour necrosis factor (TNF) to one of its receptor, TNF receptor 1 (TNFR1) induces the uptake of the activated receptor complex and the recruitment of various factors including procaspase-8 to promote apoptosis (Schneider-Brachert *et al.*, 2004). The interaction between Alix, ALG-2 and procaspase-8 has been determined in a study that also indicated their recruitment to TNFR1 containing endosomes (Mahul-Mellier *et al.*, 2008). A mutant form of Alix, unable to interact with ALG-2 was shown to prevent apoptosis, but not the endocytosis of TNFR1. The study also confirmed the findings of a preceding study that the function of Alix in apoptosis depends on its ability to interact with CMHP4B and Tsg-101, components of the ESCRT machinery (Mahul-Mellier *et al.*, 2006). Finally, the involvement of Alix in the intrinsic pathway was also suggested emphasising the importance of Alix in apoptosis (Strappazon *et al.*, 2010). Taken together these results demonstrate the crucial role of Alix in apoptosis which is highly dependent on its interactions with other molecules.

2.2.9. Cytokinesis

Cytokinesis is the last step of mitotic cell division in which the constriction of the cleavage furrow allows the separation of the two daughter cells (D'Avino *et al.*, 2015). This step can be further segmented into a few tightly regulated stages that are necessary for

symmetric division. The very final stage involves the recruitment of the ESCRT machinery to both sides of the midbody ring of the intercellular bridge, which leads to the complete division of the two daughter cells. Pioneering studies have inferred the role of Alix and Tsg-101 in this scission stage (Carlton and Martin-serrano, 2007; Morita *et al.*, 2007). The GPP motifs in the PRRs of these proteins recognise and bind CEP55, a component of the midbody ring, allowing their recruitment to the midbody. Alix and Tsg-101 then recruit CHMP4, of the ESCRT-III (Carlton *et al.*, 2008), which then polymerises to form filaments (Guizetti *et al.*, 2011). Finally, the disassembly of CHMB4 is carried out by Vps4 completing the scission process (Elia *et al.*, 2011). These studies support the imperative nature of Alix and some of the components of the ESCRT machinery in cytokinesis.

2.2.10. Brain Development

A recent work from our laboratory highlight the importance of Alix in normal development of the brain as the lack of Alix leads to microcephaly (Laporte *et al.*, 2017). The study demonstrated that Alix knockout (KO) had smaller brains when compared to wild-type brains, and that this was due to defects during neural development (Laporte *et al.*, 2017). Cortex was among the regions of the brain affected and it was suggested that imbalance of apoptotic machinery caused by the lack of Alix led to the transient activation of caspase-3 in neural progenitors, restricting the development of cortices. Furthermore, fibroblast growth factor signalling, which is crucial for the survival of telencephalic precursor cells and thus the development of the brain (Paek *et al.*, 2009), was shown to be abnormally regulated in Alix KO neural progenitor cells. It was suggested that this was due to defective CIE in these cells since Alix is a key regulator of CIE (Mercier *et al.*, 2016) and fibroblast growth factor signalling through its receptors is partly mediated by CIE (Haugsten *et al.*, 2011; Elfenbein *et al.*, 2012). Reduced dendritic arborisation and defective growth cones that were also observed in Alix KO neurons have also been implicated as causes of microcephaly. The possible causes for such observations are misregulation of the ESCRT machinery leading to changes in dendritic pruning, and defective actin dynamic and endocytosis that regulate adhesion molecules leading to the reduction of growth cones.

3. Aim of the Thesis

Taken together, it was discussed that synapses are complex structures with a variety of processes and their underlying molecular mechanisms that maintain efficient synaptic transmission. The various modes of endocytosis that regenerate SVs from the presynaptic membrane are among these processes. The different endocytic modes can be categorised into two main modes, CME and CIE. Together, they prevent presynaptic membrane expansion and decrease in SV pools.

Alix was first discovered in 1999 as an interactor of ALG-2, and the cellular processes that Alix is involved in have been discovered since then. It is clear from the studies introduced that the various functions of Alix in cells are in close association with cellular membranes. The generation of Alix KO mice by our laboratory opened doors to the discovery of new cellular processes that involve Alix and the physiological significance of the protein. Of particular interest, the recent findings that Alix depletion leads to abnormal development of brains in mice suggest an important role of Alix in the brain. One of the underlying causes of abnormal development was suggested to be defective CIE, which led to abnormal FGF signalling and reduced growth cones. The importance of Alix in CIE had already been evinced in a previous work from our laboratory.

Our laboratory also found some intriguing functional and structural abnormalities in neurons of Alix KO mice which indicated defective SV recycling in synapses. With the knowledge that Alix is a key regulator of CIE in non-neuronal cells, we deduced that Alix may take part in membrane reuptake via CIE in presynaptic boutons. The aim of this thesis is to identify the possible cause of the synaptic anomalies observed in Alix KO synapses by answering some key questions.

Results

1. Context of Study

The results are provided as an article in preparation to be submitted to the Journal of Neurosciences. I am a co-first author of the article. Continuing the work on characterizing the Alix KO mice, our laboratory has found some synaptic defects. Some of the morphological differences observed include reduced SV number and increased presynaptic terminal surface area (Figure 1 of the article). Electrophysiological studies performed by Marta Rolland (Grenoble Institute of Neuroscience, France) demonstrated that the lack of Alix leads to impairment of LTP and rapid depletion of SVs, indicating reduced pool of SVs (Figure 2 of the article). These results strongly indicated an important role of Alix in synapses. The article demonstrates for the first time that Alix (which is expressed in synapses) increases in concentration in presynaptic boutons but not postsynaptic terminals, during intense stimulation (Figure 3 of the article). This transient recruitment of Alix to presynaptic boutons during intense stimulation is accompanied by the recruitment of ALG2 and endophilin A2 (Figure 4 of the article). Finally the presence of Alix and its interactions with ALG2 and endophilin are shown to be necessary to mediate ADBE in synapses (Figure 5 of the article). The article highlights a novel role of Alix in the SV recycling.

2. Article: Alix is Required for Activity-Dependent Bulk Endocytosis at Brain Synapses

Alg-2 interacting protein-X (Alix) is required for activity-dependent bulk endocytosis at brain synapses

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Running title: Alix and ADDBE

Abstract

In chemical synapses vesicle components must be retrieved from the plasma membrane via clathrin scaffolds or via clathrin-independent processes including bulk endocytosis, in case of high frequency stimulation. Alix (ALG-2 interacting protein X)/ PDCD6IP is an adaptor protein binding to ESCRT and endophilin proteins and thereby driving deformation and fission of endosomal and cell surface membranes. We recently demonstrated that Alix, together with endophilin-A, is required for clathrin-independent endocytosis in embryonic fibroblasts of Alix knock-out mice. Here, using electron microscopy, we show that synapses from mice lacking Alix have subtle defects in presynaptic compartments, translating into flawed short-term synaptic plasticity. Using cultured neurons, we demonstrate that Alix is specifically involved in activity dependent bulk endocytosis (ADBE). We further demonstrate that in order to perform bulk endocytosis Alix must be recruited to synapses by the calcium binding protein ALG-2 and interact with endophilin-A. These results thus highlight Alix ko mice as an invaluable model to study the exact role of ADBE at synapses undergoing physiological or pathological stimulations.

Keywords: PDCD6IP, Clathrin-independent-endocytosis, Alix knock-out, endophilin-A, ALG-2, bulk endocytosis, synaptic vesicle recycling, synaptic transmission, calcium.

List of abbreviations:

4AP: 4-Aminopyridine
ABDE: Activity-Dependent Bulk Endocytosis
ALG-2: Apoptosis Linked Gene 2
Alix: ALG-2 Interacting protein X
BAPTA: 1,2-Bis(o-AminoPhenoxy)ethane-N,N,N',N'-Tetraacetic Acid
BE: Bulk Endosome
CA1: CornuAmmonis 1
CA3: CornuAmmonis 3
CHMP4B: ChargedMultivesicular body Protein 4B
CGN: Cerebellar Granule Neuron
CIE: Clathrin-Independent Endocytosis
CME: Clathrin-Mediated Endocytosis
CTxB: Cholera Toxin chain B
DIV: Day *In Vitro*
EGTA: Ethylene Glycol-bis(β -aminoethyl ether)-N,N,N',N'-Tetraacetic Acid
EM: Electron Microscope (Microscopic/ Microscopy)
ESCRT: Endosomal Sorting Complexes Required for Transport
GABAA: γ -AminoButyric Acid type-A
GFP: Green Fluorescent Protein
HRP: Horse Radish Peroxidase
kDa: kiloDalton
KO: KnockOut
LTP: Long-Term Potentiation
N-BAR: N-terminal amphipathic helix- Bin-Amphyphysin-Rvs
PRD: Proline-Rich Domain
PSD95: PostSynaptic Density Protein 95
SH3: Src Homology 3
SV: Synaptic Vesicle
Syn-pH: Synaptophysin-pHluorin
TSG-101: Tumour Susceptibility Gene-101
WT: Wild Type
YFP: Yellow Fluorescent Protein

Introduction

Neuronal communication in mammalian brain relies heavily on the activity-dependent release of chemical neurotransmitters from presynaptic boutons. Following calcium-dependent fusion of synaptic vesicles (SV) with the presynaptic membrane, SV lipids and proteins are retrieved by endocytosis. Endocytosis avoids detrimental increase in the plasma membrane surface and allows recycling of the SV components to replenish the SV pool (Gan and Watanabe, 2018). At moderate levels of stimulation, retrieval of membrane involves clathrin-mediated (CME) and clathrin-independent endocytosis (CIE) in proportions which are still highly debated (Milosevic, 2018; Soykan et al., 2016; Watanabe et al., 2013a; Watanabe et al., 2013b; Watanabe et al., 2014; Chanaday and Kanalali 2018). Moreover, long-lasting high-frequency stimulations also lead to the clathrin-independent internalization of large stretches of pre-synaptic membranes. This process, first discovered at the amphibian neuromuscular junction (Miller and Heuser, 1984) and referred to as activity dependent bulk endocytosis (ADBE), is meant to avoid abnormal increase of the synaptic bouton surface and to allow replenishment of SVs during sustained synaptic stimulations (Cheung and Cousin, 2013; Cheung et al., 2010; Marxen et al., 1999).

While most details of clathrin-dependent SV recycling have been clarified, the molecular mechanisms allowing CIE at the presynaptic membrane remain poorly understood. One actor of endocytosis at the synapse is the family of endophilin-A proteins. Endophilin-A (A1, A2 and A3) are cytoplasmic proteins which contain a N-BAR (Bin/Amphiphysin/Rvs) domain capable of sensing and generating membrane curvature, and a Src homology 3 (SH3) domain (Kjaerulff et al., 2011). Endophilin-A SH3 domains bind to proline rich domains (PRD) of dynamin and synaptojanin involved in clathrin-mediated SV endocytosis (Gad et al., 2000; Ringstad et al., 1999). Endophilin-A were also identified as major actors of CIE in fibroblasts (Boucrot et al., 2014) and have been shown to drive the fast mode of CIE at ribbon synapses (Llobet et al., 2011) as well as to mediate neck formation during ultrafast endocytosis (Watanabe et al., 2018).

We have shown that besides dynamin and synaptojanin, a major interacting partner of endophilins-A is Alix (ALG-2 interacting protein-X), first identified through its calcium-dependent binding to the penta-EF-hand protein ALG-2 (apoptosis-linked gene 2) (Chatellard-Causse et al., 2002; Missotten et al., 1999). The 95kDa cytoplasmic protein interacts with

multiple partners including CHMP4B (charged multivesicular body protein 4B) and TSG-101 (tumour suppressor gene-101), two components of ESCRT (endosomal sorting complex required for transport). Alix has been involved in numerous biological processes including programmed neuronal death, virus egress, cytokinesis and membrane repair (Bissig and Gruenberg, 2014). In each of these cases, Alix function strictly depends on its capacity to bind and recruit proteins of ESCRT complexes. More recently, we demonstrated that Alix, together with endophilin-A, was able to regulate clathrin-independent endocytosis thereby regulating both cell spreading/migration and signalisation in non-neuronal cells (Mercier, 2016 #9228; Sadoul, 2006). We, and others, also found that Alix ko mice have normally organized but smaller brains (Campos et al., 2016; Laporte et al., 2017). This microcephaly is due to a transient increase of neural progenitor apoptosis and neurite outgrowth impairments in post-mitotic neurons, both associated with an alteration of CIE (Laporte et al., 2017).

In the adult brain, Alix is ubiquitously expressed but heavily concentrated at presynaptic terminals undergoing sustained stimulation (Hemming et al., 2004). This, together with the capacity of Alix to interact with endophilin-A and its requirement for CIE, suggests a possible function of Alix in endocytosis at synapses. We found that synapse morphology and function are both altered in Alix ko brains. One obvious feature is their reduced number of synaptic vesicles and increase in size correlating with impairments in synaptic facilitation and recovery during high frequency stimulation. Using cultured neurons, we bring evidence that high frequency synaptic activity leads to calcium-dependent recruitment of ALG-2. ALG-2 in turn interacts with Alix, a necessary interaction for endophilin-A recruitment. This protein complex is indispensable for ADBE, which is selectively impaired in Alix ko neurons. Thus, our results show that some molecular mechanisms involved in ADBE may also be involved in certain aspects of synaptic plasticity such as facilitation and accommodation to synaptic fatigue.

Material and methods

Plasmids

Endophilin A2-mCherry was obtained by subcloning (In-Fusion Cloning kit, Clontech) endophilin A2 cDNA into a pmCherry-N1 vector (Clontech). GFP-ALG-2 was obtained by performing a reverse mutagenesis (Quick change II site directed mutagenesis kit, Stratagene) on a GFP-hALG2Y180A (a generous gift from Masatoshi Maki) to acquire GFP-hALG2wt.hALG2 E47A-E114A cDNA was kindly provided by Masatoshi Maki (Shibata et al., 2004) and was subcloned into a pEGFP-C1 vector (Clontech) to obtain GFP-hALG2 E47A-E114A (GFP-ALG2 Δ Ca).

All constructs containing Alix cDNA (wt or mutants) were obtained by subcloning the relevant cDNAs from pCI vectors harbouring Alix cDNA or its mutants. AlixI212D and Alix Δ PGY cDNAs in pCI were generated by mutagenesis (Quick change II site directed mutagenesis kit, Stratagene) and AlixR757E by in-fusion cloning, using the oligos given below.

mCherry-2Xflag-mAlixwt (mCherry-Alix) was obtained by subcloning 2xflag-mAlix wt cDNA into a pmCherry-C1 vector (Clontech). Alix-YFP was obtained by subcloning wild type Alix cDNA into a pEYFP-N1 vector (Clontech). GFP-flag-Alix (GFP-Alix) was described in (Mercier et al., 2016). GFP-Alix and its mutant forms (GFP-AlixR757E, GFP-Alix Δ PGY) were obtained by subcloning the various cDNAs into a pEGFP-C1 vector (Clontech). DNA constructs used for the rescue experiments were prepared in two steps. First, IRES2-GFP cDNA was subcloned into pSIN lentiviral vector (kindly provided by F. Saudou) by using pIRES2-GFP (Clontech) as a template. Then the various cDNAs were subcloned into pSIN-IRES2-GFP.

Oligos used to generate mutants:

AlixI212D (Alix Δ CHMP4B)

sense: 5'-AAGATGAAAG ATGCCGACAT AGCTAAGCTG-3'

antisense: 5'-CAGCTTAGCT ATGTCGGCAT CTTTCATCTT -3'

AlixR757E (Alix Δ endo)

sense: 5'-CAGCCGAGCC TCCACCTCCT GTGCTTCCTG -3'

antisense: 5'-GAGGCTCGGC TGGAGGCTGG GGCTTAGCAG-3'

AlixΔPGY (AlixΔALG2)

sense: 5'-GCCACAGGCT CAGGGATGCC AAATGCCCAT GC-3'

antisense: 5'-GCATGGGCAT TTGGCATCCC TGAGCCTGTG GC -3'.

Antibodies

| Antibody | Supplier (reference) | Species (type) | Dilution |
|--------------------------------------|--------------------------------------|---------------------|----------|
| Anti-Actin | Millipore (MAB1501R) | Mouse (monoclonal) | 1/10000 |
| Anti-Alix | Covalab (ab0204) | Rabbit (polyclonal) | 1/10000 |
| Anti-phospho-p44/42 MAPK (T202/Y204) | Cell signaling (9106) | Mouse (monoclonal) | 1/1000 |
| Anti-PSD95 | NeuroMab (73-028) | Mouse (monoclonal) | 1/2000 |
| Anti-PSD95 | Millipore (MAB1598) | Mouse (monoclonal) | 1/500 |
| Anti-Synapsin-1 | Millipore (AB1543P) | Rabbit (polyclonal) | 1/1000 |
| Anti-Synaptophysin | Merck Millipore (MAB5258) | Mouse (monoclonal) | 1/5000 |
| Anti-Flag | Sigma-Aldrich (F3165) | Mouse (monoclonal) | 1/1000 |
| Anti-Flag | Sigma-Aldrich (F7425) | Rabbit (polyclonal) | 1/1000 |
| Anti-Mouse HRP | Jackson ImmunoResearch (115-035-166) | Goat (polyclonal) | 1/5000 |
| Anti-Rabbit HRP | Jackson ImmunoResearch (115-035-044) | Goat (polyclonal) | 1/5000 |
| Anti-Mouse Alexa Fluor 488 | Invitrogen (A-11029) | Goat (polyclonal) | 1/1000 |
| Anti-Mouse Alexa Fluor 594 | Invitrogen (A-11032) | Goat (polyclonal) | 1/1000 |
| Anti-Mouse Cy5 | Jackson ImmunoResearch (115-175-146) | Goat (polyclonal) | 1/500 |
| Anti-Rabbit Alexa Fluor 488 | Invitrogen (A-11034) | Goat (polyclonal) | 1/1000 |
| Anti-Rabbit Alexa Fluor 594 | Invitrogen (A-11037) | Goat (polyclonal) | 1/1000 |
| Anti-Rabbit Cy5 | Jackson ImmunoResearch (111-175-144) | Goat (polyclonal) | 1/1000 |

Animals

Animals were handled and killed in conformity with European law and internal regulations of INSERM. Pregnant Oncins France souche A (OFA; a substrain of Sprague Dawley) rats (Charles River) and C57BL/6 mouse pups Alix wt and Alix ko (Laporte et al., 2017; Mercier et al., 2016) were used for primary neuronal culture. One to two month-old C57BL/6 Alix wt and Alix ko mice were used for electrophysiological recordings, histochemistry and electron microscopy studies. Mice were anesthetized by intraperitoneal injection of 0.1 ml sodium pentobarbital (5.6% w/v; CEVA Sante' Animale) and treated as described in the corresponding sub-

headings of the material and method section.

Cell culture and transfection

Cortical and hippocampal neurons from rat E18 embryos and P0 Alix wt and ko mice were prepared as previously described (Faure et al., 2006). Briefly, cortices and hippocampi were dissected from E18 rat embryos or P0 pups, treated with trypsin, and mechanically dissociated. Dissociated cells were seeded at a density of $5 \times 10^4/\text{cm}^2$ in 100 mm dishes for cortical neurons and $1.5 \times 10^4/\text{cm}^2$ onto acid-washed coverslips (either 13 mm diameter or 25 mm; Marienfeld) in either 4-well plates or P35 dishes (ThermoScientific) precoated for 4 h with 50 $\mu\text{g}/\text{ml}$ poly-D-lysine (Sigma). Neurons were maintained in neuronal culture medium (Neurobasal medium containing 2% B27 supplement, 10 unit/mL penicillin, 10 $\mu\text{g}/\text{mL}$ streptomycin, and 0.5 mM L-glutamine; Invitrogen) supplemented with 10% heat-inactivated horse serum (Invitrogen). Neurons were maintained in water-saturated 95% air/5% CO_2 at 37°C. The seeding medium was replaced after 20 h with serum-free neuronal culture medium.

Neurons were transfected at 10 DIV as previously described (Chassefeyre et al., 2015). Briefly, for each P35 dish, 2 μg plasmid DNA, 250mM CaCl_2 were mixed with equal volume of 2x BES-buffered saline and left to precipitate for 20 min at room temperature. Neurons were placed in transfection medium (Minimum Essential Medium containing 0.22% NaHCO_3 , 20mM D-glucose and 0.5mM L-glutamine) supplemented with 2% B27, before the DNA precipitate was added. They were then incubated for 1.5 hat 37°C and 5% CO_2 . Neurons were then washed by being placed in transfection medium (pre-warmed at 37°C in 10% CO_2) for 20 min at 37°C and 5% CO_2 . Finally, they were transferred back into their conditioned medium.

Primary cultures of cerebellar granule neurons (CGN) were prepared from 6-day-old C57BL/6 Alix wt and Alix ko pups as described previously (Trioulier et al., 2004), with some modifications. The cerebella were removed, cleared of their meninges, and cut into 1-mm pieces. They were then incubated at 37°C for 10 min in 0.25% trypsin-EDTA and DNase (1500 U/mL). Trypsin was inactivated and cells were dissociated in culture medium (DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 25 mM KCl, 10 mM HEPES and 10 unit/ml penicillin, 10 $\mu\text{g}/\text{ml}$ streptomycin). After filtration on 70 μm cell strainers, neurons were plated at $5 \cdot 10^5$ cell/ cm^2 onto poly-D-lysine (10 $\mu\text{g}/\text{ml}$, Sigma) precoated coverslips.

Cytosine- β -D-arabinoside (10 μ M, Sigma) was added after 1 day in vitro (DIV) to prevent the growth of non-neuronal cells until 8DIV when neurons were used for activity dependent bulk endocytosis experiment (see below).

Golgi staining

2 month-old anesthetized mice were dislocated prior to brain dissection and 100 μ m thick cortical brain sections were cut on a vibratome. The dendritic spines of hippocampal neurons from the CA1 *stratum radiatum* were visualized by the Golgi impregnation technique. For this, we used the FD Rapid GolgiStain kit (FDNeuroTechnologies). Brain sections were immersed in equal volumes of solutions A and B for 7d and impregnated with solution C for 48 h at 4°C. Then, the sections were washed twice in double-distilled water and incubated for 10 min in a mixture of one part of solution D, one part of solution E, and two parts of double-distilled water. Sections were washed twice, dehydrated with increasing concentrations of ethanol, and mounted with epoxy resin (Fluka) between two coverslips. Stacks of bright-field images with 0.3 μ m spacing were acquired with a Zeiss Axioskop 50 microscope with 63x oil objective (NA 1.4; Plan-Apochromat) coupled to a CCD camera (CoolSnap ES; Roper Scientific) operated by Metaview software (Molecular Devices). Images were analysed with ImageJ. The number of spines per unit dendritic length was counted.

Electron microscopy

2 month-old anesthetized mice were intra-cardially perfused with phosphate-buffered 0.9% NaCl (PBS), followed by 0.1 M phosphate buffered 4% paraformaldehyde, pH 7.4, supplemented with 0.05% glutaraldehyde (Sigma). The brains were carefully removed, postfixed for 4 h in the same fixative and 60 μ m sections were cut with a vibratome. After several washes in PBS, the sections were postfixed in 1% glutaraldehyde in the same buffer for 10 min and processed for EM. This included treatment with osmium tetroxide (1% in 0.1 M PB), block staining with uranyl acetate, dehydration through a graded series of ethanol, and flat embedding on glass slides in Durcupan (Fluka) resin. Regions of interest were cut at 70 nm on an ultramicrotome (Reichert Ultracut E; Leica) and collected on one-slot copper grids. Staining was performed on drops of 1% aqueous uranyl acetate, followed by Reynolds's lead citrate. EM images were acquired in a JEOL-1200 electron microscope with a digital camera (Veleta, SIS; Olympus) and analysed with ImageJ. Twenty images per animal from 3

animals per genotype were used for quantification. A synapse was considered if it met 3 criteria: a presynaptic bouton filled with at least 10 synaptic vesicles (1) juxtaposed to the head of a dendritic spine with a clearly visible PSD (2) and the presence of the neck in the section (3). Numbers of synaptic vesicles and areas of presynaptic boutons were quantified in each synapse using the free-shape tool and the cell counter plugins of ImageJ. We used the straight tool of ImageJ to measure the lengths of PSDs, and head and neck diameters. Note that the head diameter was taken parallel to the PSD and the neck diameter was perpendicular to the neck membranes.

Immunofluorescence

Cultured hippocampal neurons were fixed for 20 min at room temperature in phosphate-buffered 4% paraformaldehyde supplemented with 4% sucrose. After three washes in PBS, cells were permeabilized and blocked in PBS containing 0.3% Triton X-100 and 3% BSA for 15 min at room temperature. Coverslips were incubated for 1–2 h at room temperature with primary antibodies diluted in the blocking solution. After washing in PBS, cells were incubated for 1 h with secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 594, or Cyanine 5 (Cy5), diluted in the blocking solution. Coverslips were rinsed and mounted in Mowiol. Images were acquired on a Leica SPE microscope using a 40x dry objective (NA 0.75, Leica) or a 100x oil immersion objective (NA 1.4, Leica) at 488 nm, 532 nm or 635 nm.

Synapse density: Cultured hippocampal neurons were fixed and stained with antibodies at 14–16 DIV as described above. Presynaptic boutons (synapsin-1-positive) and postsynaptic terminals (PSD-95-positive) were selected using ‘Spot Detector’ (wavelet detection with size filtering between approximately 0.4 μm and 2 μm in diameter) on max image projections. Synapses were defined as spots of colocalization between the detected presynaptic and postsynaptic terminals that were within approximately 3 μm of each other. Synapses were counted using ‘Colocalization Studio’ of ICY software.

Protein recruitment assays: Cultured hippocampal neurons were transfected with expression vectors 24~48 h prior to stimulation. The transfected neurons were incubated in basal medium (150 mM NaCl, 5 mM KCl, 1.3 mM CaCl_2 , 10 mM HEPES and 33 mM D-glucose at pH 7.4) for 10 min prior to stimulation. Coverslips were either treated with basal medium alone or bicuculline/4AP solution (basal medium supplemented with 100 μM bicuculline and 5 mM 4-aminopyridine) for 5 min at 37°C and 5% CO_2 . Coverslips were fixed and stained with

antibodies against synapsin-1 and PSD95 as described above. Images were acquired on a Leica SPE microscope using a 100x oil immersion objective (NA 1.4, Leica) at 488 nm, 532 nm or 635 nm. The acquired images were analysed using ImageJ. Regions of interest were drawn on areas of axons which colocalize with anti-synapsin-1 spots, but not with anti-PSD95 spots to ensure presynaptic measurement. Fluorescence intensities were then measured in these regions of interest and normalised to fluorescence intensities measured on other regions of axons to give the relative fluorescence intensity at presynaptic boutons.

Live Fluorescence Imaging of Alix recruitment to synapses

For live imaging of protein recruitment, cultured hippocampal neurons were co-transfected with either mCherry-Alix or endophilin-A-mCherry and synaptophysin-pHluorin 2~4 days prior to imaging. All live imaging experiments were performed at 37°C and images were acquired using a spinning disk confocal microscope (AxioObserver Z1) with a 63x oil objective (NA 1.46, Zeiss) at 488 nm and 561 nm excitation. Transfected neurons were placed in basal medium for 10 min and then mounted in an imaging chamber (POC-R2 Cell cultivation system, Zeiss). Imaging lasted 12 min and consisted of 2 min in basal medium, 5 min in bicuculline/4AP solution and 5 min in basal medium. A 5x bicuculline/4AP solution was added (final concentration, 100 μ M and 5 mM, respectively) for stimulation which lasted 5 min and the chamber was perfused with basal medium at 3 ml/min for washing. Regions of interest were drawn on 'presynapses' defined by spots of synaptophysin-pHluorin that increased during stimulation. For both synaptophysin-pHluorin and mCherry-Alix, fluorescence values were measured in these regions of interest and then normalised to the initial fluorescence values (fluorescence values prior to stimulation) using ImageJ.

Ex vivo slice preparation

Brain slices were prepared from 2 month-old C57BL/6 wt and Alix ko mice. The brains were removed quickly and 350 μ m-thick sagittal slices containing both cortex and hippocampus were cut in ice-cold sucrose solution (2.5 mM KCl, 1.25 mM NaH₂PO₄, 10 mM MgSO₄, 0.5 mM CaCl₂, 26 mM NaHCO₃, 234 mM sucrose, 11 mM glucose, saturated with 95% O₂ and 5% CO₂) with a Leica VT1200 blade microtome (Leica Microsystems, Nanterre, France). After cutting, hippocampi were extracted from the slice and transferred to oxygenated Artificial Cerebro-Spinal Fluid (ACSF: 119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 1.3 mM MgSO₄,

2.5 mM CaCl₂, 26 mM NaHCO₃, 11 mM glucose) at 37± 1°C for 30 min and then kept at room temperature for at least 1 h before recordings.

Electrophysiological recordings in CA1

Each slice was individually transferred to a submersion-type recording chamber and continuously superfused (2ml/min) with oxygenated ACSF. Extracellular recordings were obtained at 28°C from the apical dendritic layers of the hippocampal CA1 area, using glass micropipettes filled with ACSF. Field excitatory postsynaptic potentials (fEPSPs) were evoked by the electrical stimulation of Schaeffer collaterals afferent to CA1. The magnitude of the fEPSPs was determined by measuring their slope. Signals were acquired using a double EPC 10 Amplifier (HEKA Elektronik Dr. Schulze GmbH, Germany) and analysed with Patchmaster software (HEKA Elektronik Dr. Schulze GmbH, Germany).

Input/output: The slope of fEPSPs was plotted as a function of stimulation intensity (30 to 60 µA).

Paired-pulse facilitation (PPF): PPF of synaptic transmission was induced by paired-pulse stimulation with an inter-stimulus interval of 50 ms. PPF was quantified by normalising the second response to the first one.

Frequency facilitation: Schaffer collaterals were stimulated repetitively by 25 stimuli of the same intensity at 25Hz.

Long-term potentiation: Test stimuli were delivered once every 15s and the stimulus intensity was adjusted to produce 40-50% of the maximal response. A stable baseline was recorded for at least 15 min. LTP was induced by high frequency stimulation (4 trains delivered at 100 Hz with 5 min between each train). Average value of fEPSP slope was expressed as a percentage of the baseline response ± SEM.

Patch-clamp experiments: hippocampal pyramidal neurons from CA1 were visualized in a chamber on an upright microscope with transmitted illumination and continuously perfused at 2 ml/min with an oxygenated ACSF at room temperature. Miniature excitatory postsynaptic currents (sEPSC) were recorded in presence of tetrodotoxin (TTX, 1µM) and bicuculline (50 µM) and at a membrane potential of -60 mV with borosilicate glass pipettes of 4-5 MΩ resistance filled for whole-cell recordings (117.5 mM CsMeSO₄, 15.5 mM CsCl, 10 mM TEACl, 8 mM NaCl, 10 mM HEPES, 0.25 mM EGTA, 4 mM MgATP, 0.3 mM NaGTP, pH 7.3). Signals were acquired using a double EPC 10 Amplifier (HEKA Elektronik Dr. Schulze GmbH,

Germany) filtered at 2 kHz, sampled at 10 kHz and analysed with Patchmaster software (HEKA Elektronik Dr. Schulze GmbH, Germany). Recordings were considered stable when the input and access resistances did not change more than 20% during the experiment.

Synaptosomal preparation from cortical neurons

Synaptosome-enriched membranes from 15 DIV cortical neurons were prepared as described previously with some modifications (Frandemiche et al., 2014). Briefly, cultured neurons were stimulated for 15 min with a mixture of bicuculline/4AP (50 μ M/2.5 mM). After a wash in HBSS, neurons were homogenized by passing 15-20 times through 0.25G needle in cold buffer containing 0.32 M sucrose, 10 mM HEPES, 15mM NaF, 15mM β -glycerophosphate and protease inhibitors (Roche), pH 7.4. Samples were maintained at 4°C during all steps of the experiment. Homogenates were cleared at 1000g for 10 min to remove nuclei and large debris. The resulting supernatants were spun down at 12,000g for 20 min to obtain a crude membrane fraction and washed twice in HEPES buffer 4 mM containing 1 mM EDTA, 15 mM NaF, 15 mM β -glycerophosphate and protease inhibitors (Roche), pH 7.4. The resulting pellet was solubilized in Triton (0.5% Triton X-100, 20 mM HEPES, 100 mM NaCl, 15mM NaF, 15 mM β -glycerophosphate and protease inhibitors, pH 7.2) for 20 min at 4°C with mild agitation and analysed by Western blot.

Western blot

Cells lysates were resuspended in Laemmli buffer and resolved by SDS-PAGE in 10% polyacrylamide gels. Proteins were electrotransferred onto PVDF membranes that were then blocked for 30 min in TBS containing 0.1% Tween 20 and 5% dry milk and incubated for 1 h to overnight with primary antibodies diluted in the blocking solution. After washes in TBS–Tween, the membranes were further incubated for 1 h with secondary antibodies coupled to HRP, washed as before and incubated with luminescence-generating HRP substrate. Bound antibodies were revealed by luminography on film.

Imaging of synaptophysin-pHluorin upon electrical stimulation

Hippocampal neurons from Alix wt and ko mice were transfected with synaptophysin-pHluorin (Syp-pH) at 6 DIV by a calcium phosphate transfection procedure. The recycling of synaptic vesicles was imaged in a buffer solution containing 120 mM NaCl, 5 mM KCl, 2

mM CaCl₂, 2 mM MgCl₂, 5 mM glucose, 10 mM HEPES adjusted to pH 7.4 and 270 mOsmol/l. Experiments were carried out at 34°C. Neurons were stimulated by electric field stimulation (platinum electrodes, 10 mm spacing, 1 ms pulses of 50 mA and alternating polarity at 5-40 Hz) applied by a constant current stimulus isolator (SIU-102, Warner Instruments). The presence of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM D,L-2-amino-5-phosphonovaleric acid (AP5) prevented recurrent activity.

Experiments were performed on an inverted microscope (IX83, Olympus) equipped with an Apochromat N oil 100× objective (NA 1.49). Images were acquired with an electron multiplying charge coupled device camera (QuantEM:512SC; Roper Scientific) controlled by MetaVue7.1 (Roper Scientific). Samples were illuminated by a 473-nm laser (Cobolt). Emitted fluorescence was detected after passing a 525/50 nm filter (Chroma Technology Corp.). Time-lapse images were acquired at 1 or 2 Hz with integration times from 50 to 100 ms.

Image analysis was performed with custom macros in Igor Pro (Wavemetrics) using an automated detection algorithm as described previously (Martineau et al., 2017). The image from the time series showing maximum response during stimulation was subjected to an “à trous” wavelet transformation. All identified masks and calculated time courses were visually inspected for correspondence to individual functional boutons. The intensity values were normalized to the ten frames before stimulation. All data are represented as mean ± SEM of n experiments.

Quantification of calcium increase during stimulation

Hippocampal neurons cultured on 24-well plates were placed in basal medium and incubated with 1 μM Fluo-4-AM for 1 h at 37°C. After washing, neurons were left in 250 μl basal medium for 5 min. Plates were then transferred to a Pherastar automatic plate reader (BMG Labtech, Germany) set to 37 °C, to record fluorescence intensity at 0.1 Hz during 590 s, for each well, and with fluorescence measurement settings as described on <http://www.bmg-labtech.com/media/35216/1043854.pdf>. Neurons were stimulated with 50 μl 6x bicuculline/4AP solution (final concentration, 100 μM and 5 mM, respectively) after 290 s with automatic injection.

Quantification of ADBE by Dextran uptake

The protocol for dextran uptake was adapted from (Clayton and Cousin, 2009b). 15-17 DIV hippocampal neurons were stimulated with Bicuculline/4AP solution for 5 min, at 37°C and 5% CO₂, in the presence of 10 kDa tetramethylrhodamine-dextran (50 μM). Coverslips were immediately washed several times in washing solution (basal medium supplemented with 0.2% BSA and warmed to 37°C) to remove excess dextran. For ADBE inhibition, neurons were stimulated in the presence of 2 μM GSK3 inhibitor (CT99021, Tocris) and placed in fresh basal medium containing 2 μM GSK3 inhibitor for 10 min at 37°C and 5% CO₂. Neurons were then fixed as previously described and imaged with a Leica SPE microscope using a 40x dry immersion objective (NA 0.75, Leica) at 532 nm excitation. Analysis was performed on ImageJ. The number of fluorescent spots was counted in a defined field of view (130 μm x 130 μm) in thresholding analysis with a diameter limit between 300 nm and 2 μm (resolution limit for the microscope and maximum size of a nerve terminal).

Rescue experiments: Cultured hippocampal neurons were transfected 2 to 4 d prior to the day of experiment. Dextran uptake assay was performed as described above. Images were acquired on a Leica SPE microscope using a 40x oil immersion objective (NA 1.25, Leica) at 488 nm and 532 nm excitation. The analysis was performed on ICY software. Regions of interest were generated closely around axons by using 'thresholder'. Then dextran spots within these regions of interest were counted by using 'spot detector' with size limit between 300 nm and 2 μm. The length of axon per field of view was estimated by manually drawing 'Polyline type ROI' over the axon images. The number of dextran spots per μm of axon was calculated and expressed as ratio to control values.

Quantification of ADBE by EM

Analysis of ADBE from cerebellar granule neurons was performed as described previously with some modifications (Cheung et al., 2010). 8DIV CGN were pre-incubated in hyperpolarizing medium (170 mM NaCl, 3.5 mM KCl, 0.4 mM KH₂PO₄, 20 mM TES, 5 mM NaHCO₃, 5 mM D-glucose, 1.2 mM Na₂SO₄, 1.2 mM MgCl₂, 1.3 mM CaCl₂, pH7.4) for 10 min prior to stimulation. Neurons were then incubated for 2 min with 10 mg/ml HRP (Sigma P8250) in either the hyperpolarizing medium or a hyper-potassic solution containing 50 mM KCl (123.5 mM NaCl, 50 mM KCl, 0.4 mM KH₂PO₄, 20 mM TES, 5 mM NaHCO₃, 5 mM D-glucose, 1.2 mM Na₂SO₄, 1.2 mM MgCl₂, 1.3 mM CaCl₂, pH7.4) before rapid washing in PBS

and fixation in PBS-glutaraldehyde 2% for 30 min. After three washes in Tris buffer 100 mM, endocytosed HRP was revealed by incubation in Tris 100 mM containing 0.1% diaminobenzidine and 0.2% H₂O₂. The cultures were then post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon. Synapses were photographed with a JEOL-1200 electron microscope.

Statistical analysis

The comparison of two groups was performed using a Student's t-test or its non-parametric correspondent, the Mann-Whitney test, if normality was not granted either because not checked or because rejected (Shapiro-Wilks test). The comparisons of more than two groups were made using one or two ways ANOVAs followed by post-hoc tests (Holm Sidak's or Tukey's HSD, see table 1 for details) to identify all the significant group differences. N indicates independent biological replicates. The graphs with error bars indicate 1 SEM (+/-) except for figure 1D-I where we used box plots showing distribution of median (whiskers = min and max values). The significance level is denoted as usual (*p<0.05, **p<0.01, ***p<0.001). All the statistical analyses were performed using Prism7 (Graphpad version 7.0a, April 2, 2016). Means, intervals of confidence, degrees of freedom and p values were calculated using Prism7. However, Effect sizes are either those reported by the software or Cohen's D calculated as the difference of means divided by the pooled weighted standard deviation : $(|m1 - m2|) / \sqrt{((SD1^2 \times \frac{n1}{(n1+n2)}) + (SD2^2 \times \frac{n2}{(n1+n2)}))}$

The results of the statistical analysis are listed in Table 1.

Results

Alix is required for synaptic plasticity

We have previously reported that Alix ko animals suffer from primary microcephaly in part due to a defect in dendrite outgrowth (Laporte et al. 2017). Here, we found that the density of dendritic spines in the CA1 hippocampal region of adult brains stained with Golgi-Cox was not different between wt and Alix ko animals (Figure 1A). Similarly, electron microscopy examination of the same CA1 hippocampal region revealed no difference in the number of synaptic contacts between wt and Alix ko neurons (Figure 1B). Finally, the number of synapses in cultured hippocampal neurons revealed by co-immunostaining with pre- and postsynaptic markers, synapsin-1 and PSD95, respectively, were similar between wt and Alix ko (Figure 1C). Thus, Alix does not seem to be required for synaptogenesis to occur. We next quantified synapse morphology using electron micrographs in the CA1 *stratum radiatum*. We found that Alix ko synapses contain significantly less synaptic vesicles (SV) than synapses in wt synapses (Figure 1D, E). Furthermore, the size of postsynaptic densities, which is known to be strictly correlated with the number of SV (Harris & Stevens, 1989), was similarly reduced in Alix ko synapses (Figure 1G, H). Importantly, the surface of Alix ko synaptic boutons was also significantly increased suggesting plasma membrane accumulation in these synapses (Figure 1D, F). Finally, at the postsynaptic level, we also noticed that the ratio between the diameter of the spine head and that of the neck was changed in Alix ko animals suggesting possible defects in maturation or plasticity of adult synapses lacking Alix (Figure 1G, I).

We next examined if the morphological differences detected in Alix ko synapses might translate into alterations of their function using electrophysiological recording of acute hippocampal slices from adult animals. We performed field recordings in the CA1 *stratum radiatum* of hippocampal slices during stimulation of Schaffer collaterals. As shown on figure 2A the input-output curves were not significantly different between Alix ko and wt animals indicating that, in this region, both the connectivity and the basal synaptic transmission are not grossly affected by the lack of Alix. However, in the same slices, long-term potentiation (LTP) induced by tetanic stimulations was significantly impaired in Alix ko, compared to wt hippocampal slices (average recordings between 25 and 35 min post-high-frequency stimulation: 105.02 ± 13.86 % in Alix ko compared to 180.70 ± 11.19 % in wt, Figure 2B). This defect in potentiation might be related to presynaptic anomalies detected in the same

CA3/CA1 synapses. Indeed, the paired-pulse facilitation ratio was significantly reduced in ko animals (1.43 ± 0.04 in ko vs 1.62 ± 0.07 in wt, Figure 2C). Furthermore, patch clamp recording of CA1 pyramidal neurons revealed that while the amplitude of spontaneous postsynaptic currents was unchanged (-56.89 ± 2.68 for ko and -57.55 ± 4.65 for wt, Figure 2D), their frequency, which correlates with the number of presynaptic vesicles, was reduced by almost half in ko animals (0.21 ± 0.018 in ko and 0.11 ± 0.024 in wt, Figure 2E). Finally, synaptic fatigue, which reflects depletion of the SV pool induced by repeated stimulations at 25 Hz, was significantly increased in absence of Alix (Figure 2F).

Thus, decrease in the frequency of spontaneous postsynaptic currents, in facilitation and in the capacity to recover during repeated stimulations in Alix ko synapses correlate well with the lower number of SVs observed by EM (Figure 1D, E) and highlight Alix as a central regulator of the genesis of SVs.

Alix is recruited to synapses upon synaptic activation.

We next used dissociated cortical neuron cultures to decipher the role of Alix at synapses. Western blot analysis during *in vitro* differentiation revealed that Alix expression strongly increases during synaptogenesis as indicated by the parallel rise in postsynaptic density protein 95 (PSD95) expression (Figure 3A). Moreover, synaptosome-enriched membranes prepared from cortical neurons (15 DIV) contained Alix (Figure 3B). This synaptic pool increased when neuron cultures were incubated 15 min with the GABA_A receptor antagonist bicuculline, together with a weak potassium channel blocker 4-aminopyridine (4AP) (Figure 3B). As such short term treatments lead to high frequency glutamatergic synaptic activity (Hardingham et al., 2001), this observation suggests that Alix tends to concentrate at synapses during their activation. To show that this is indeed the case, we used live imaging to follow mCherry-Alix relocalization to synapses in 15 DIV hippocampal neurons. While the fluorescent signal was homogeneously distributed throughout the entire neuronal cytoplasm in resting conditions (Figure 3C, t= 0 min), it increased by about two-fold in discrete spots within neuron processes during synaptic activation (arrowhead Figure 3C, t= 2, 4 and 6 min; Movie 1) corresponding to active presynaptic boutons labelled with Synaptophysin-pHluorin (Syp-pH). Alix recruitment to these sites is concomitant with the activation of glutamatergic synapses, as it became detectable few seconds after addition of bicuculline/4AP to the culture medium and decreased soon after washing the cells (Figure 3D). Thus we

demonstrate that mCherry-Alix recruitment occurs at presynaptic sites that display transient increase of pHluorin fluorescence induced by stimulation (arrowheads Figure 3C, Figure 3D; Movie 1). Alix localization at axonal boutons was confirmed in bicuculline/4AP stimulated and fixed-neurons expressing Alix-YFP and immunolabelled for synapsin-1 (Figure 3E, F). Similar experiments using anti-PSD95 immunostaining showed that Alix positive spots were juxtaposed to, but did not overlap with the postsynaptic PSD marker, further demonstrating that Alix recruitment during synaptic activation occurs presynaptically (Figure 3G, H).

In non-neuronal cells, Alix recruitment to the plasma membrane wounds was shown to be caused by calcium entry (Scheffer et al., 2014). In neurons, action potential depolarization induces a massive and transient accumulation of calcium in the bouton, which triggers fusion of SV with the plasma membrane. Calcium increase seems necessary for Alix recruitment at active synapses since addition of the intra-cellular calcium chelators BAPTA-AM or EGTA-AM completely abolished Alix-YFP recruitment (Figure 4A). One partner of Alix is ALG-2, a penta-EF-hand containing protein, which binds calcium and thereafter interacts with Alix. In non-neuronal cells, ALG-2 was found to be required for the calcium dependent recruitment of Alix at the plasma membrane (Scheffer et al., 2014). Interestingly, GFP-ALG-2 concentrated at presynaptic boutons upon synaptic activation, in contrast to an ALG-2 point-mutant unable to bind calcium (ALG-2 Δ Ca) (Figure 4B, C). ALG-2 recruitment does not depend on Alix, as it concentrates at stimulated synapses of both wt and Alix ko neurons (Figure 4B, 3Dleft bars). On the contrary, Alix recruitment to active synapses is tightly dependent on its capacity to interact with ALG-2, as a mutated version of the protein unable to interact with ALG-2 (Alix Δ ALG-2) (Suzuki et al., 2008; Trioulier et al., 2004) does not accumulate at presynaptic boutons upon stimulation (Figure 4D, right bars).

Among other partners of Alix, endophilins-A are main regulators of endocytosis at synapses and impact the number of SVs (Ringstad et al., 2001; Schuske et al., 2003). We therefore tested if endophilins could be recruited similarly to Alix and ALG-2 at active synapses. Indeed, endophilin-A2-mCherry fluorescence at presynaptic bouton increased upon glutamatergic activation (Figure 4E, F; Movie 2). Remarkably, no such increase could be seen in Alix ko neurons, whereas Alix deleted of its endophilin binding domain (Alix Δ endo), was still able to be recruited at synapses upon activation (Figure 4G). This observation strongly suggests that endophilins are recruited by Alix at active synapses.

Altogether, our observations indicate that calcium increase at depolarized synapses allows

the transient recruitment of ALG-2/Alix and endophilin-A at the presynaptic membrane. Because of our EM observations showing that Alix ko synapses are bigger and have significantly less SVs, we next tested which step of the turnover of presynaptic vesicles might be affected by the lack of the protein. We used Syp-pH expressing neurons and followed their fluorescence during electrical stimulation to trigger exocytosis. Recapture of synaptic proteins from the plasma membrane results in decay in fluorescence, reflecting SV retrieval and vesicle re-acidification (Soykan et al., 2017). As illustrated in figure 5A,B, the kinetics of fluorescence show an increase during stimulation (exocytosis) followed by decrease thereafter (endocytosis), which do not dramatically differ between Alix ko and wt neurons for two stimulation frequencies, 5 and 40 Hz. However, the time constant of the exponential decay was slightly decreased in Alix ko neurons stimulated at 40 Hz suggesting a higher rate of Syp-pH endocytosis (Fig.5B). Noteworthy, this apparent increase in SV endocytosis was only detected after 40 Hz stimulation.

Based on Alix role in fluid phase endocytosis demonstrated in Alix ko fibroblasts, we next tested if the protein might also intervene in ADBE at synapses. One well described system to study clathrin-independent ADBE is cultured cerebellar granular neurons (CGN) depolarized with 50 mM KCl for two minutes to induce long-lasting high frequency depolarization. While being depolarized, cells are incubated with Horse Radish Peroxidase (HRP), which is endocytosed and labels vesicles and endosomes as they form. In Alix wt synapses, depolarization dramatically increased the number of vacuoles decorated by HRP that apparently had undergone fission from the plasma membrane and were therefore identified as bulk endosomes (Figure 5C, D, red arrowheads). Other HRP positive vesicles having the size of neurotransmitter vesicles were also more numerous in depolarized synapses (Figure 5C, D, blue arrowheads). In Alix ko synapses, the number of depolarization induced bulk endosomes was strongly reduced (Figure 5 D) while the number of newly formed SV was significantly increased, suggesting a mechanism compensating for ADBE deficiency (Figure 5E). This is consistent with the increased endocytic rate detected by Syp-pH in Alix ko neurons stimulated at 40 Hz (Fig. 5B).

Another way to assess for ADBE is by the use of fluorescent 10kDa dextran, a fluid phase cargo that accumulates inside bulk endosomes but fails to label vesicles formed by clathrin-dependent mechanisms upon neuronal stimulation (Clayton and Cousin, 2009a).

Hippocampal neurons incorporated dextran when incubated in presence of bicuculline/4AP. A GSK3 inhibitor, known to block ADBE but not other modes of SV endocytosis (Clayton et al., 2010), completely abolished the dextran labelling (Figure 5F, G). In contrast, Alix ko neurons failed to endocytose dextran (Figure 5H, I) even though bicuculline/4AP stimulation induced a calcium entry equivalent in both Alix ko and wt neurons (Figure 5J). Interestingly, neurons also failed to endocytose dextran when treated with calcium chelators BAPTA and EGTA (Figure 5K), both blocking Alix recruitment to presynaptic boutons (Figure 4A). Finally, rescue experiments showed that the impairment in ADBE observed in Alix ko cells is due solely to the absence of Alix, since restoring Alix expression fully restored the capacity of Alix ko cells to endocytose 10kDa dextran (Figure 5L). In sharp contrast, expression of Alix Δ ALG-2 as well as Alix Δ Endo was not able to rescue dextran endocytosis by Alix ko neurons (Figure 5L). Noteworthy, an Alix mutant (Alix Δ 4B) unable to bind the ESCRT-III protein CHMP4B, was still able to rescue ADBE in Alix ko neuron. These results demonstrate that the capacity of Alix to drive ADBE requires interaction with both endophilin-A and ALG-2 but not with ESCRT-III.

In summary, Alix is specifically required for ADBE. This endocytic function could be related to the lower number of SV and increase in the surface of presynaptic membranes in ko neurons observed *in vivo* as well as impairments in synaptic function.

Discussion

To sustain neurotransmission and prevent expansion of the presynaptic plasma membrane, SV fusion is coupled to the endocytic recycling and regeneration of SV proteins and lipids. Vesicle components can be retrieved from the plasma membrane via clathrin scaffolds or via clathrin-independent processes like ultrafast and fast endocytosis or, in case of high frequency stimulation, bulk endocytosis (Gan and Watanabe, 2018). Using Alix ko cells, we recently discovered that, together with endophilins, Alix drives clathrin-independent-endocytosis (CIE) (Laporte et al., 2017; Mercier et al., 2016). Here, in agreement with a role of Alix in endocytosis at synapses, we report that Alix ko mice have fewer SVs than normal and are defective in short term plasticity. Using hippocampal cultures, we also found that synaptic hyperactivity leads to the presynaptic recruitment of Alix with two of its interactors, endophilins and ALG-2. Furthermore, impairment in ADBE was obvious in Alix ko neurons and could not be rescued when Alix capacity to interact with ALG-2 or endophilin was impaired.

We have previously reported that Alix immunoreactivity is strongly upregulated in hippocampal synapses undergoing massive activation during kainate-induced epileptic seizures in the rat. Interestingly, this increase was presynaptic and only transient, being reversed soon after cessation of the seizures (Hemming et al., 2004). In order to reproduce and study the possible role of Alix recruitment to presynaptic parts seen during seizures, we used cortical and hippocampal cultures, which contain glutamatergic neurons and inhibitory interneurons. The latter impose a tonic inhibition on the neuronal network because GABA_A receptor blockage by bicuculline causes the neurons to fire synchronous bursts of action potentials. Adding 4-aminopyridine (4-AP), a weak potassium channel blocker, to bicuculline markedly increases burst frequency and results in elevated calcium entry (Hardingham et al., 2001). We found that bicuculline/4AP induced bulk endocytosis, which was completely blocked by intracellular calcium chelators. It also induced Alix to transiently concentrate at firing synapses in a calcium-dependent way. Consistent with the kainate-induced Alix increase seen during epileptic seizures, relocalization of the protein concerned exclusively presynaptic parts. Interestingly, EGTA, which binds calcium at an approximately 100 times slower on-rate than BAPTA (Adler et al., 1991; Schneggenburger and Neher, 2005), was equally efficient as BAPTA in blocking ADBE as well as inhibiting Alix recruitment. Since EGTA cannot prevent formation of microdomain calcium at the active zone, this suggests that

recruitment of Alix to synapses requires calcium outside of microdomains linked to SV exocytosis. In line with this, the kinetic of the increase in Syp-pH fluorescence, which underscores SV exocytosis, was not different between wt and Alix ko neurons stimulated with 200 action potential at low (5Hz) or high frequency (40 Hz). However, the decline in the pHluorin signal reflecting reacidification of the endocytosed vesicles was slightly faster in Alix ko neurons suggesting an increase in SV endocytosis only visible at 40 Hz. At these frequencies, this approach has been shown to reflect a form of CIE endocytosis (Kononenko *et al.*, 2014) different from ADBE which requires either higher frequency or prolonged stimulation (Wenzel *et al.*, 2012). Thus, our results show that Alix is not involved in SV endocytosis at hippocampal synapses. However, ADBE revealed by dextran uptake was severely impaired in Alix ko hippocampal neurons stimulated with bicuculline/4AP. EM observations confirmed this impairment in Alix ko cerebellar granule neurons.

The coupling of exo- and endocytosis is assumed to be Calcium-dependent through mechanisms which remain unknown (Wu *et al.*, 2014). ADBE occurs outside of the active zone and is triggered by high $[Ca^{2+}]$ in response to sustained activity (Paillart *et al.*, 2003). The calcium and calmodulin-dependent phosphatase calcineurin was suggested to make a link between synaptic activity and formation of bulk endosomes through dynamin (Morton *et al.*, 2015). Alix may represent another link as its recruitment and activity in ADBE seems mediated by the calcium-binding protein ALG-2. ALG-2, is a cytosolic penta-EF hand-containing-protein with two Ca^{2+} binding sites ($K_d = 1.2 \mu M$), and its interaction with Alix strictly depends on calcium (Missotten *et al.*, 1999; Shibata *et al.*, 2004; Trioulier *et al.*, 2004). Conformational change and exposure of hydrophobic patches occur at μM concentrations of Ca^{2+} , suggesting that ALG-2 functions as a calcium sensor (Maki *et al.*, 2016). Indeed, in non-neuronal cells, calcium entry provoked by membrane wounds leads to the sequential recruitment to the membrane of ALG-2, Alix and ESCRT-III proteins necessary for membrane repair (Jimenez *et al.*, 2014; Scheffer *et al.*, 2014). In neurons, the activity-dependent accumulation of ALG-2 at synapses was totally abolished by point-mutations within the two Ca^{2+} binding sites. Furthermore, ALG-2 recruitment at synapses did not require Alix, as it also occurred in Alix ko neurons. Finally, Alix deleted of its ALG-2 interacting domain (Alix Δ ALG-2) was unable to relocalize to synapses and did not rescue ADBE in Alix ko neurons. Thus, our observations highlight cytosolic ALG-2 present at synapses as an obvious candidate for sensing calcium elevation and suggest the following scenario: high frequency depolarization

leads to massive elevation of calcium in the synaptic bouton; Ca^{2+} binding to ALG-2 allows its accumulation at the synaptic membrane and binding to cytosolic Alix, whose recruitment to the membrane drives ADBE. It remains unclear how ALG-2 interacts with lipid bilayers (Scheffer et al., 2014), but Alix changes conformation upon binding to ALG-2 and is known to interact with lysobisphosphatidic acid (LBPA), a lipid normally present in late endosomes (Bissig and Gruenberg, 2014; Bissig et al., 2013; Sun et al., 2015).

Little is known about the molecular mechanisms underlying the plasma membrane deformation during ADBE. The proline rich domain of Alix interacts with the SH3 domains of endophilins-A1-3 which contain N-BAR domains and have been shown to regulate clathrin-dependent and -independent SV endocytosis at different synapses (primarily A1 and A3 in mammals) (Gad et al., 2000; Llobet et al., 2011; Ringstad et al., 1999). BAR domains are dimerization domains able to induce, stabilize and sense membrane curvature (Farsad et al., 2001; Kjaerulff et al., 2011). Interestingly a proteomic approach has recently revealed the presence of endophilin-A1 in bulk endosomes (Kokotos et al., 2018). Furthermore, endophilin-A2, actin and dynamin were found to mediate a restricted type of clathrin-independent endocytosis activated upon ligand binding to cargo receptors (Boucrot et al., 2014; Renard et al., 2014). Using Alix ko fibroblasts, we were able to show that endophilin and Alix act in the same CIE pathway, even if Alix is more promiscuous for ligands as it is also involved in fluid phase endocytosis. While Alix and endophilin were independently recruited to extracellular ligand-induced endocytic sites, their interaction seems to favor their recruitment or stabilize complexes at the membrane (Mercier et al., 2016). In activated synapses, Alix is necessary for the synaptic recruitment of endophilin-A. However, Alix recruitment does not require interaction with endophilins. Endophilin-A binding to the membrane occurs through their BAR domains, which recognize concave cytoplasmic surfaces of the plasma membrane, as in the case of membrane sites bent by extracellular ligands (Boucrot et al., 2014; Kjaerulff et al., 2011). At membranes of synapses under high calcium elevation, Alix might be the adaptor needed to recruit endophilin to membranes that are not yet curved in order to support membrane deformation necessary for ADBE. The lack of rescue of ADBE by an Alix point mutant unable to interact with endophilin is reminiscent of the situation seen in the case of CIE of CTxB (Mercier et al., 2016) and demonstrates that the Alix-endophilin complex is required for ADBE. Interestingly, the incapacity of Alix to interact with ESCRT-III, known to alleviate its plasma membrane repair ability (Jimenez et al., 2014;

Scheffer et al., 2014), did not alter rescue of ADBE. This result also discriminates Alix-driven ADBE from roles of the ESCRT system in the degradation of SV proteins (Sadoul et al., 2017; Sheehan et al., 2016).

ADBE occurs at high frequency stimulation and the fact that Alix concentrates at rat hippocampal synapses during kainate-induced epileptic seizures, reinforces the idea that ADBE is mainly activated during pathological situations (Cousin, 2009). Strikingly however, Alix ko animals were characterized by a subtle but significant decrease in the number of SVs, together with obvious impairment in short-term plasticity and LTP. Since Alix mediates only ADBE without apparently affecting other modes of SV endocytosis, our observations suggest that ADBE might also be necessary for regulating normal synaptic physiology and plasticity. They also highlight Alix ko mice as an invaluable tool for exploring and understanding more precisely the exact role of ADBE at synapses undergoing normal and pathological stimulations.

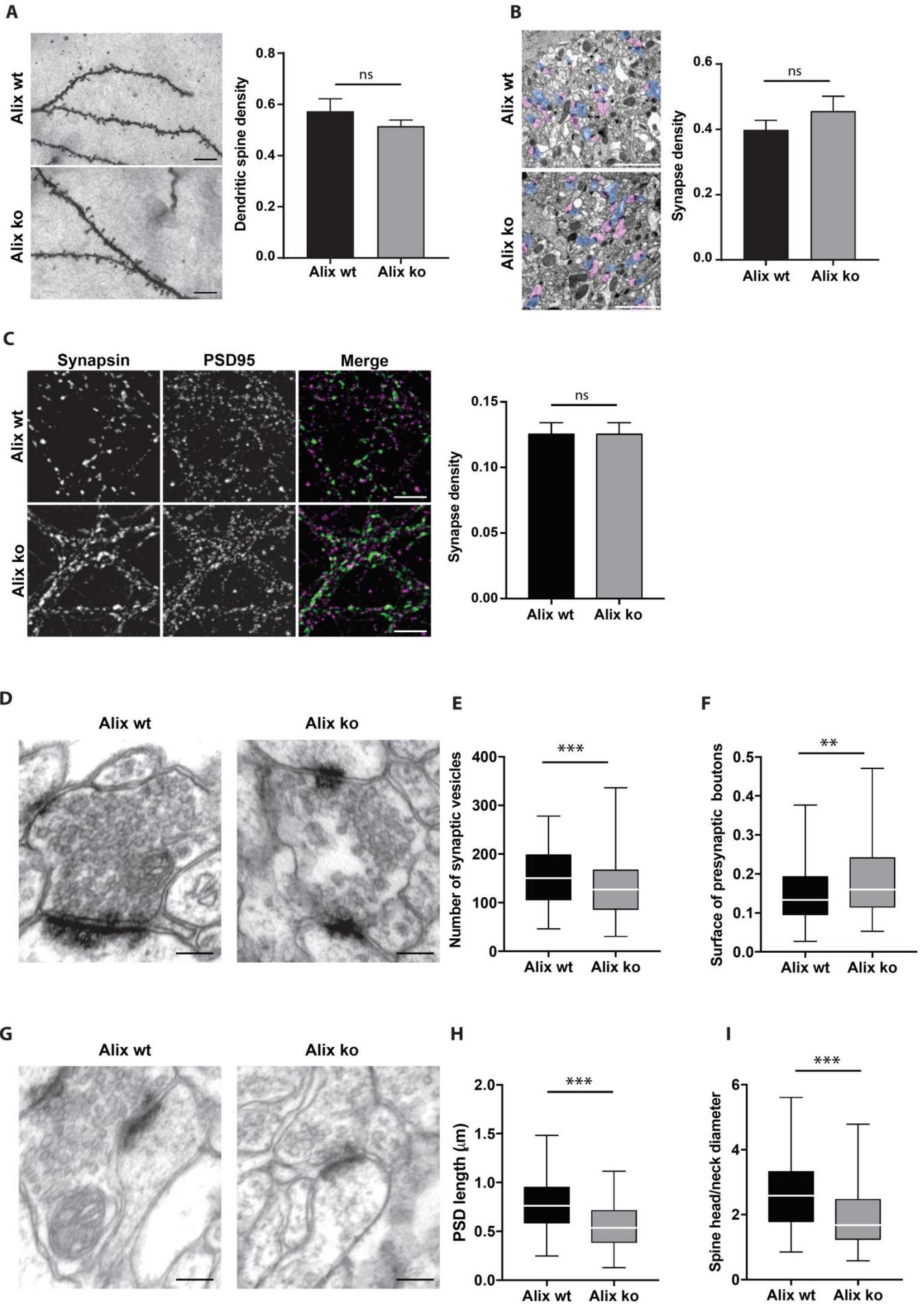


Figure 1. Alix is required for synaptic organisation. **A)** Brain sections from 8-week-old Alix ko or littermate controls (Alix wt) were stained by the Golgi-Cox impregnation technique. Stained dendritic segments were visualized by bright-field microscopy (scale bar: 10 μm). Numbers of spines per μm of dendrites were counted (n=3 animals per genotype, p=0.1414, Unpaired t test). **B)** Representative electron micrographs of the CA1 from Alix wt and ko mice. Presynaptic profiles are highlighted in blue, and dendritic spines are in purple (scale bar: 2 μm). Graph shows the synaptic density per μm^2 (n=3 animals per genotype, p=0.1395, Unpaired t test). **C)** 15 DIV hippocampal neurons were stained by dual immunofluorescence with anti-PSD-95 and anti-synapsin-1 antibodies. Immunolabelled objects were considered synapses when both stainings were juxtaposed (scale bar: 10 μm). The graph represents the number of synapses per μm^2 (n=7 experiments, p=0.5501, Unpaired t test). **D, G)** Representative electron micrographs of CA1 from Alix wt and ko mice (scale bar: 200 nm). Graphs represent numbers of synaptic vesicles per μm^2 (**E**, n=136 synapses from 3 animals, p=0.0009, Mann Whitney test), presynaptic bouton surface area (**F**, n=136 synapses from 3 animals, p=0.0036, Mann Whitney test), PSD length (**H**, n=136 synapses from 3 animals, p=0.0001, Mann Whitney test) and ratio between the diameter of the spine head and neck (**I**, n=136 synapses from 3 animals, p=0.0001, Mann Whitney test).

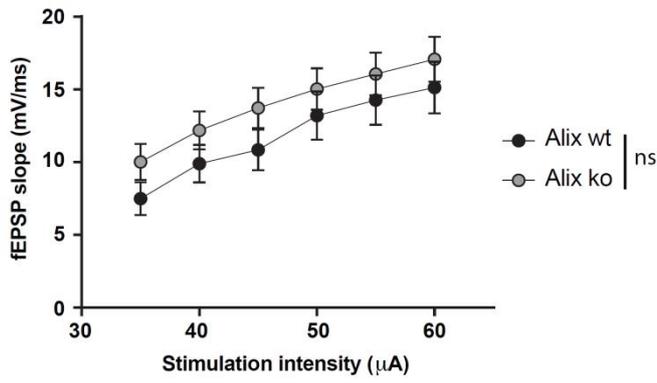
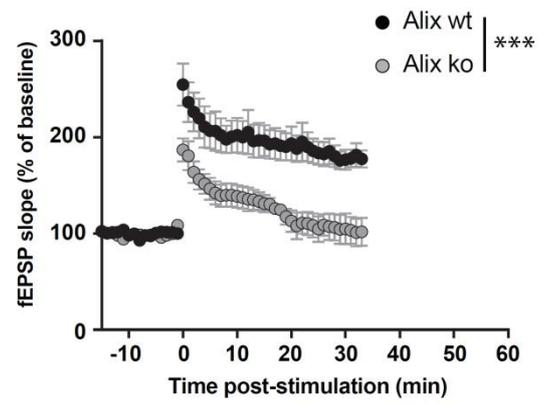
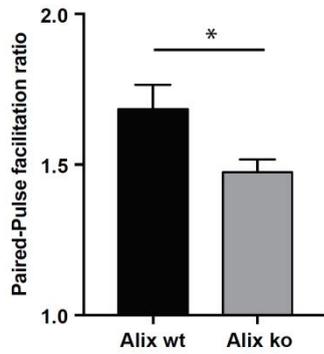
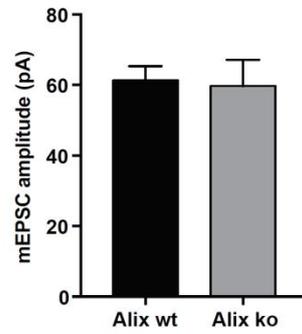
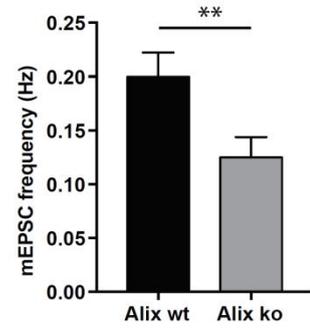
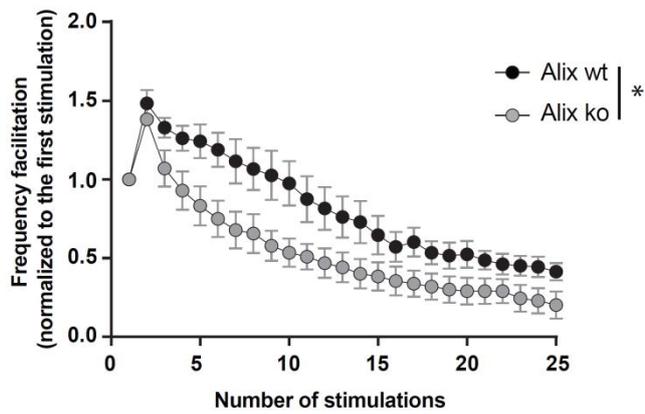
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Figure 2. Synaptic plasticity in CA1 is altered in absence of Alix. **A)** Input-output relationship of fEPSP slope evoked by stimulation of Schaeffer collaterals at various intensities in slices from Alix wt and ko (n=16 wt slices and n=13 ko slices from 3 animals per genotype, $p=0.9166$, two-way ANOVA). **B)** Long-term potentiation of fEPSP slope evoked by high frequency stimulation of Schaeffer collaterals delivered at time 0 (n=5 slices from 3 animals per genotype, $p=0.0001$, two-way ANOVA). **C)** Paired-pulse facilitation ratio. Facilitation is induced by two consecutive stimuli of Schaeffer collaterals separated by 50ms (n=16 wt slices and n=13 ko slices from 3 animals per genotype, $p=0.0187$, Unpaired t test). **D, E)** Quantification of the amplitude (**D**) and the frequency (**E**) of spontaneous miniature excitatory postsynaptic currents recorded in CA1 pyramidal neurons (n=15 wt slices and n=10 ko slices from 5 animals per genotype, $p=0.2609$ for D and $p=0.0063$ for E, Mann Whitney test). **F)** Synaptic fatigue induced by stimulation of Schaeffer collaterals at 25Hz (n=5 slices from 3 animals per genotype, $p=0.0001$, two-way ANOVA).

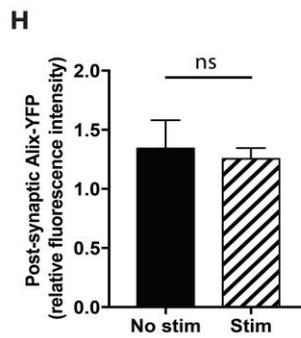
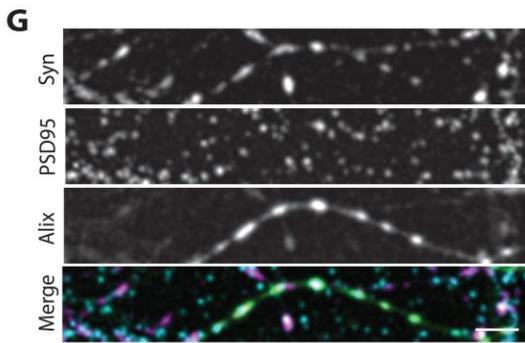
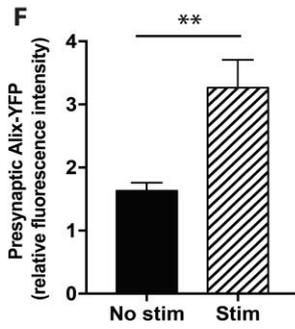
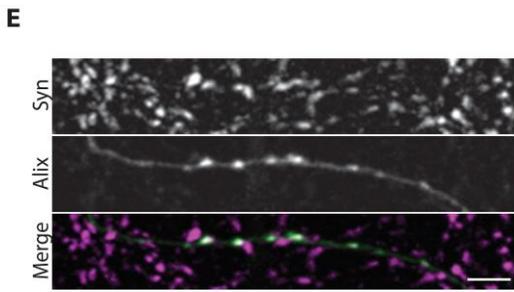
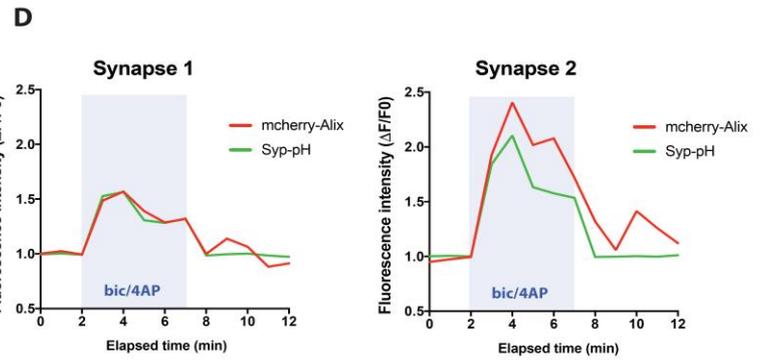
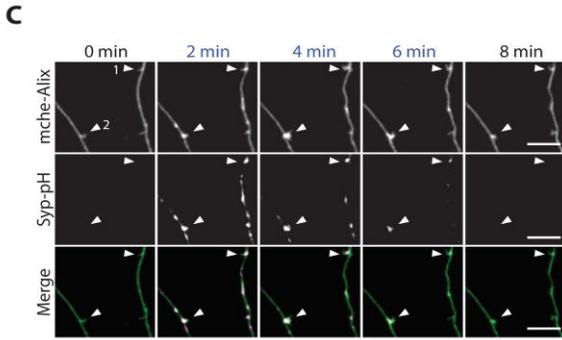
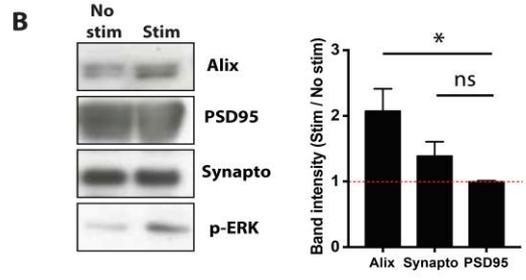
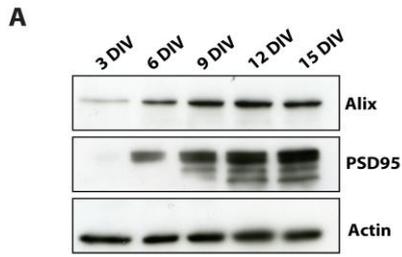


Figure 3. Alix is recruited presynaptically during synaptic activation. **A)** Western blot showing Alix expression during neuronal maturation in cortical cultures. PSD95 expression was used to monitor synaptogenesis with time in culture. **B)** Western blot showing increase of Alix in synaptosome-enriched neuronal membranes upon neuronal stimulation by bicuculline/4AP. Synaptophysin and PSD95 were used as pre- and postsynaptic markers, respectively. The phosphorylated form of ERK (p-ERK) was used to assess the efficiency of the stimulation. (n=4 experiments, Alix versus PSD95, p=0.0187, one-way ANOVA). **C)** Images from time-lapse video microscopy of hippocampal neurons expressing mCherry-Alix (mche-Alix) and synaptophysin-pHluorin (Syp-pH). White arrowheads indicate presynaptic boutons where Alix is recruited during bicuculline/4AP stimulation (images at 2, 4 and 6 min)(scale bar: 10 μ m). **D)** Profile of mche-Alix recruitment to presynaptic boutons 1 and 2 of figure 3C and the corresponding Syp-pH profile. Blue line indicates the bicuculline/4AP incubation. **E, F)** 15 DIV hippocampal neurons expressing Alix-YFP (green) stimulated for 5 min, fixed and presynaptic boutons revealed with anti-synapsin-1 antibody (Syn, magenta) (scale bar: 5 μ m). Graph shows the presynaptic increase in Alix-YFP upon stimulation. Presynaptic Alix-YFP corresponds to the ratio of YFP fluorescence between synapsin-positive and -negative axonal regions (n=12 neurons per condition from 4 experiments, p=0.0017, Unpaired t test). **G, H)** 15 DIV hippocampal neurons expressing Alix-YFP (green), stimulated for 5 min, fixed and stained with anti-PSD95 (cyan) for postsynaptic densities and anti-synapsin-1 (magenta) (scale bar: 5 μ m). Graph shows no recruitment of Alix in dendritic spines upon stimulation. Postsynaptic Alix-YFP corresponds to the ratio between YFP-fluorescence at PSD95 labelled ROI and at neighbouring dendritic parts (n=33 and n=25 neurons for both conditions, from 5 experiments, p=0.3821, Mann Whitney test).

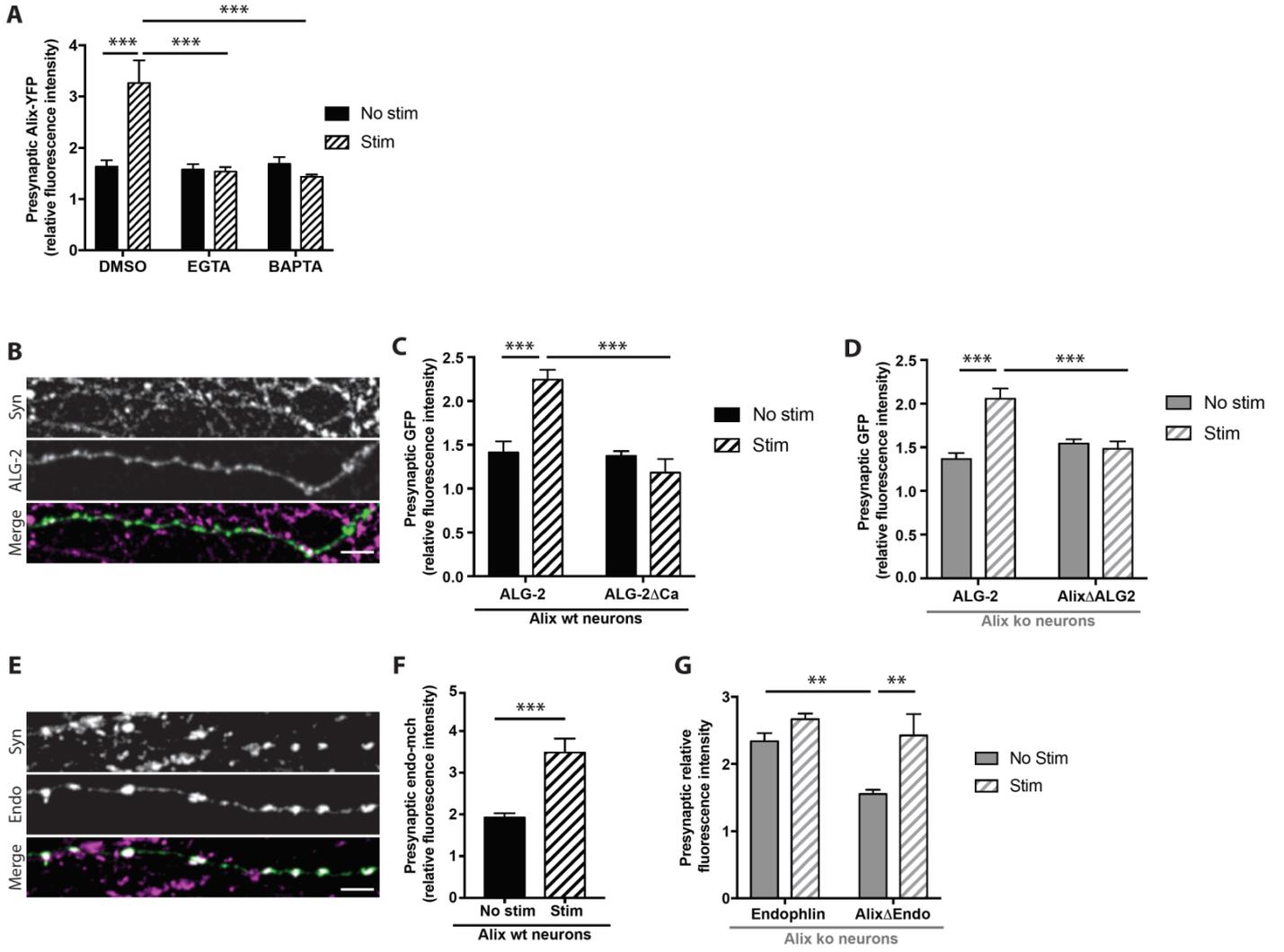


Figure 4. Calcium regulates Alix recruitment and its partner endophilin-A. **A)** Calcium chelators (BAPTA-AM and EGTA-AM) block presynaptic Alix recruitment upon stimulation. Presynaptic Alix-YFP corresponds to the ratio of fluorescence between presynaptic and non-synaptic axonal ROI (n=12, 9 and 9 neurons for DMSO, EGTA and BAPTA respectively. $p=0.0001$, one-way ANOVA). **B, C)** ALG-2 recruitment depends on its capacity to bind calcium. 15 DIV wt hippocampal neurons expressing GFP-ALG2 (green) or GFP-ALG2 Δ Ca (magenta), stimulated for 5 min, fixed and stained with anti-synapsin-1 (magenta) (scale bar: 5 μ m). Graph shows the presynaptic recruitment of GFP-ALG2 but not GFP-ALG2 Δ Ca. Presynaptic GFP is the ratio of fluorescence between synapsin-positive and -negative axonal regions (n=10 and n=7 neurons for GFP-ALG2 stimulated and not stimulated, respectively and n=6 and n=12 neurons for GFP-ALG2 Δ Ca stimulated and not stimulated, respectively, in 3 experiments, $p=0.0001$, one-way ANOVA). **D)** Presynaptic recruitment of GFP-ALG2 but not GFP-Alix Δ ALG2 in Alix ko neurons. Presynaptic GFP corresponds to the ratio of fluorescence between presynaptic and non-synaptic regions (n=17 neurons for both GFP-ALG2 stimulated and not stimulated, and n=15 and n=18 neurons for GFP-Alix Δ ALG2 stimulated and not stimulated, respectively, from at least 3 experiments, $p=0.0001$, one-way ANOVA). **E, F)** endophilin-mCherry (endo-mch, green) expressed in 15 DIV wt hippocampal neurons concentrates at synapsin-positive synapses during 5 min stimulation (scale bar: 5 μ m). Presynaptic mCherry corresponds to the ratio of fluorescence between synapsin-positive and -negative axonal regions (n=12 and n=10 neurons for stimulated and not stimulated, respectively from 3 experiments, $p=0.0001$, Mann Whitney test). **G)** Presynaptic recruitment GFP-Alix Δ endo but not of endophilin-mCherry in Alix ko neurons. Presynaptic fluorescence corresponds to the ratio of fluorescence between presynaptic and axonal regions (n=15 and n=13 neurons for endophilin-mCherry stimulated and not stimulated, respectively, and n=9 and n=10 neurons for GFP-Alix Δ endo stimulated and not stimulated, respectively, from 3 experiments $p=0.0031$, one-way ANOVA).

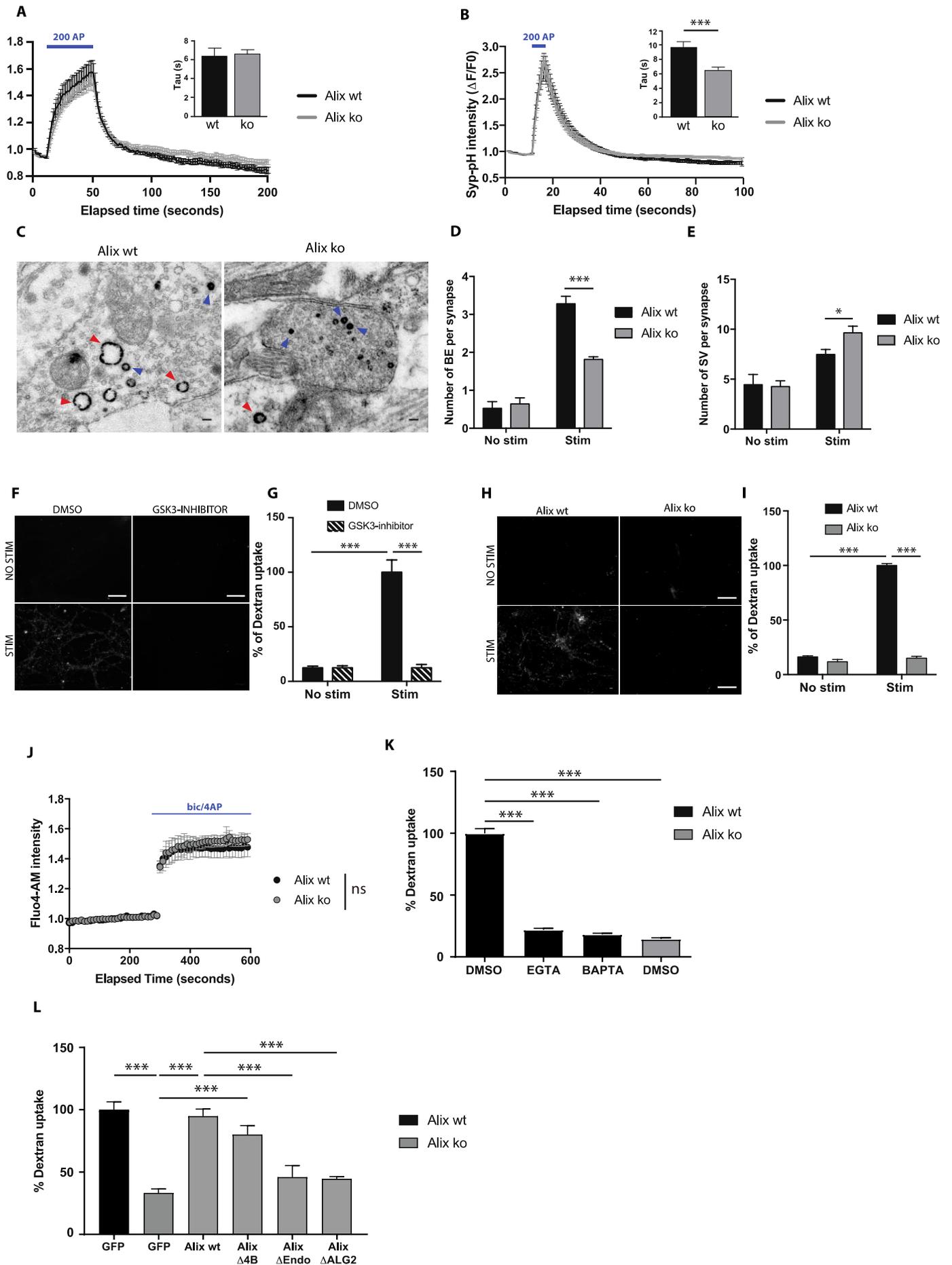


Figure 5. Alix is necessary for activity-dependent bulk endocytosis. **A, B)** Average traces of Syp-pH fluorescence in synaptic boutons of Alix wt and ko hippocampal neurons stimulated with 200 action potentials (AP) applied at 5 Hz (A) or 40 Hz (B) (16-46 fields of view for each condition in 4 experiments for wt and 5 for ko mice). Insets show the average \pm SEM of exponential fit of fluorescence decay after stimulations in the fields imaged. (A) $p = 0.99$; (B) $p = 0.001$ one-way ANOVA. **C)** Electron micrographs of Alix wt and ko cerebellar granule neurons stimulated in presence of free HRP to label newly-formed synaptic vesicle (blue arrowhead) and bulk endosomes (red arrowhead) (scale bar: 100 nm). **D, E)** comparison of the number of bulk endosomes (D) and synaptic vesicles (E) in Alix wt and ko cerebellar neurons (**D**, $n=4$ experiments, $p=0.0008$, one-way ANOVA) or synaptic vesicles (**E**, $n=4$ experiments, $p=0.0393$ one-way ANOVA). **F, G)** Confocal images of Alix wt hippocampal neurons stimulated in the presence of 10kDa dextran with or without a GSK3 inhibitor (scale bar: 50 μ m). Dextran uptake is abolished in Alix wt neurons treated with an inhibitor of bulk endocytosis (GSK3-inhibitor). % dextran uptake corresponds to the number of dextran spots per ROI expressed as percentages of the positive control ($n=3$ experiments, $p=0.0001$, one-way ANOVA). **H, I)** Confocal images of Alix wt and Alix ko hippocampal neurons stimulated in the presence of 10kDa dextran (scale bar: 50 μ m). Dextran uptake occurs in stimulated Alix wt, but not in Alix ko neurons. ($n=4$ experiments, $p=0.0001$, one-way ANOVA). **J)** No difference in calcium rise in Alix wt and ko hippocampal neurons upon bicuculline/4AP stimulation. Fluo4-AM intensity corresponds to the change in fluorescence, normalized to initial fluorescence ($n=4$ experiments, $p=0.9999$, two-way ANOVA). **K)** Dextran uptake is abolished in wt neurons by calcium chelators BAPTA and EGTA. % dextran uptake corresponds to the number of dextran spots per ROI expressed as percentages of the positive control for each experiment ($n=3$ experiments, $p=0.0001$, one-way ANOVA). **L)** Dextran uptake is rescued in Alix ko neurons expressing Alix wt and Alix Δ Chmp4B (Alix Δ 4B), but not Alix Δ ALG2 or Alix Δ endo. % of dextran uptake corresponds to the number of dextran spots per μ m expressed as percentages of the positive control for each experiment ($n=17$ neurons for wt neurons, $n=13$ neurons for ko+GFP, $n=11$ for ko+Alix, $n=12$ neurons for ko+Alix Δ endo, $n=12$ for ko+Alix Δ Chmp4B, and $n=11$ for ko+Alix Δ ALG2, all from at least 3 experiments, $p=0.0001$, one-way ANOVA).

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Additional Results

1. Protein recruitment is limited to a subpopulation of synapses.

Regarding protein recruitment assays, we noticed that not all synapses display increases in fluorescence. This suggests that the three different proteins examined may recruit to a subpopulation of synapses. To test this, we first set a threshold value at 4 standard deviations above the mean fluorescence intensity measured at synapses of unstimulated neurons. This threshold value was determined due to variability within the 'no stimulation' group. Fluorescence intensity values were considered significant if they were greater than the threshold. Indeed a subpopulation of all the synapses analysed displayed significant recruitment of the three proteins (37% of all analysed synapses showed significant recruitment of Alix, 28.8% for ALG-2 and 39.4% for endophilin-A).

| Percentage of synapses displaying significant recruitment | | | | | |
|---|--------------------------|------------------------------|--------------------------|------------------------------|---------------------------|
| Alix | | Endophilin-A | | ALG-2 | |
| No stim (n=350 synapses) | Stim (n=527 synapses) | No stim (n=1162 synapses) | Stim (n=991 synapses) | No stim (n=1313 synapses) | Stim (n=1198 synapses) |
| 0.3% | 37.0%* | 8.5% | 39.4%* | 0.9% | 28.8%* |

Table 3. Alix, ALG-2 and Endophilin-A are recruited to a subpopulation of synapses.

Percentage of synapses displaying significant increase in fluorescence intensity (n=indicated number of synapses from at least 3 experiments each). *, $p < 0.001$ using a chi-square test to test the difference between 'not stimulated' and 'stimulated' conditions for each protein.

2. Synaptophysin-pHluorin Response Indicates Reduced SV Exocytosis in the Presence of EGTA and BAPTA

We examined the effects of the intracellular calcium chelators (EGTA-AM and BAPTA-AM) on synaptophysin-pHluorin response during Bic/4AP stimulation. Decreases in synaptophysin-pHluorin responses were observed in EGTA-AM and BAPTA-AM treated neurons (figure 18A). We then calculated area under the curves (AUCs) of the first and second stimulations and divided the values to obtain 'AUC stim2 / AUC stim1'. Significant reductions were observed in this ratio (figure 18B) indicating a reduction in SV exocytosis in neurons treated with the calcium chelators.

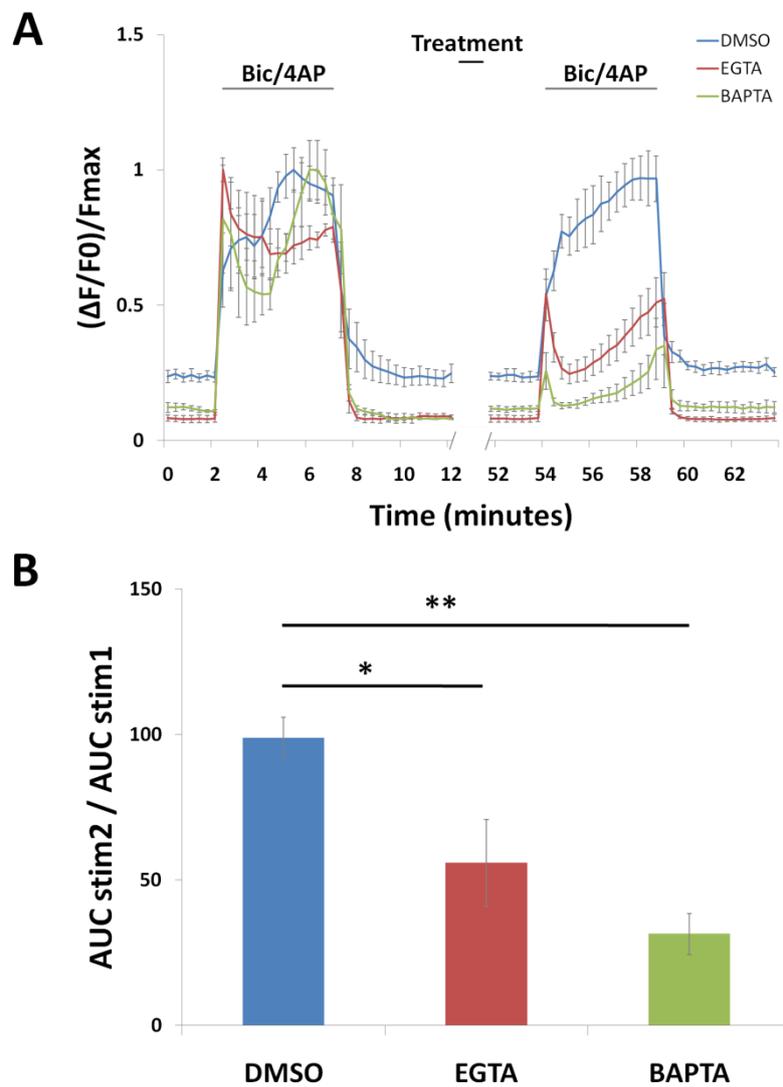


Figure 18: Change in presynaptic fluorescence indicates recruitment of mCherry tagged Alix and endophilin to presynaptic boutons, but not mCherry alone

Cultured hippocampal neurons (from wild-type mouse pups at postnatal day 0) were pre-incubated with 0.01% DMSO for 30 minutes at 37°C and 5% CO₂. Then the first imaging was carried out for 12 minutes during which the neurons were stimulated for 5 minutes with Bic/4AP. After a 10 minute rest, the neurons were incubated with 0.01% DMSO, 100µM EGTA-AM or 100µM BAPTA-AM for 30 minutes. Then the second imaging was carried out in the same manner as the first. **A, B**) Reduction in synaptophysin-pHluorin response in neurons treated with EGTA-AM and BAPTA-AM (n=4 neurons for DMSO and EGTA, n=3 neurons for BAPTA. DMSO versus EGTA p=0.0209, DMSO versus BAPTA p=0.0063, one-way ANOVA). 'AUC stim2/AUC stim1' was obtained by calculating the area under the curves (AUCs) during the first and second stimulations separately, then dividing AUC of the second stimulation by the first.

3. Fluorescence Increase in Presynaptic Boutons During Intense Stimulation Do Not Represent Injury-Induced Varicosities

We noticed that the increase in fluorescence at presynaptic boutons resemble varicosities that form in axons as a result of injury due to excitotoxicity or mechanical stress, which is also known as axon beading (Gu *et al.*, 2017). This was likely in our live imaging system as a perfusion system was used which allowed the flow of solutions at 2~3ml/min. To test whether the increase in fluorescence at presynaptic boutons truly represents increased protein concentration or merely axon beading due to axon damage, we compared the fluorescence changes in neurons transfected with mCherry-Alix, endophilin-A2-mCherry or mCherry alone. While fluorescence increase in presynaptic boutons was observed in neurons transfected with mCherry tagged Alix or endophilin-A2 during intense stimulation, no increase was observed in neurons transfected with mCherry alone (figure 19).

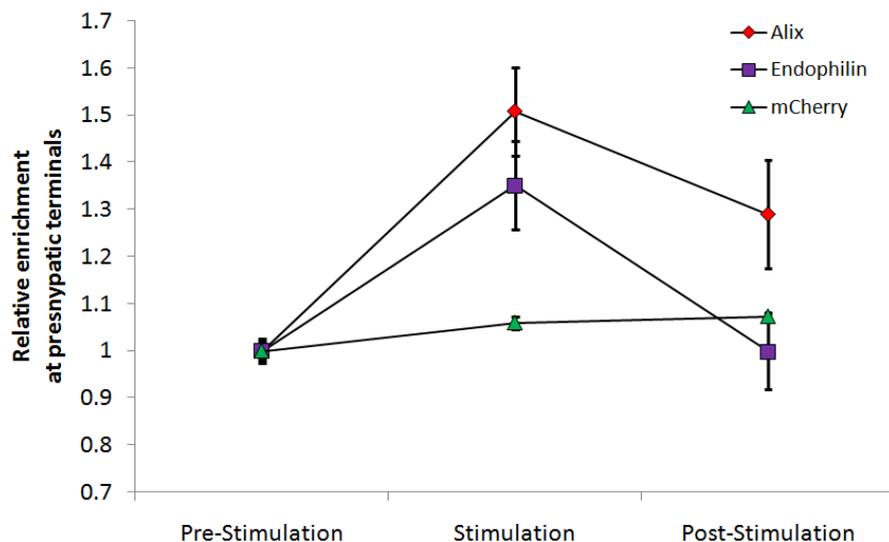


Figure 19: Change in presynaptic fluorescence indicates recruitment of mCherry tagged Alix and endophilin to presynaptic boutons, but not mCherry alone

Average fluorescence values of mCherry-Alix, endophilin-A2-mCherry and mCherry alone calculated from live imaging. All fluorescence values were averaged in 3 groups: pre-stimulation, stimulation and post-stimulation. Recruitment (reported by increase in fluorescence intensity) is observed for Alix and endophilin, but not mCherry.

Discussion

Efficient synaptic transmission underlies healthy functioning of the central nervous system and SV recycling is at the core of this process. Synapses have a range of different SV recycling modes that allow the regeneration of SVs in the dynamic environment of synapses. The first discovery of SV recycling dates back around 40 years (Heuser and Reese, 1973), but the molecular mechanisms that underpin remain elusive, especially in light of the recent controversies that seem to refute the pioneering works.

Recent work from our laboratory showed a novel role of Alix in CIE in fibroblasts; a process which involves endophilin, a major interacting partner of Alix (Mercier *et al.*, 2016). The ability of Alix to mediate CIE may explain some aspects of the abnormalities seen in Alix KO mouse brain (Laporte *et al.*, 2017). Our laboratory further investigated the Alix KO brain and discovered some synaptic defects that suggested improper SV recycling.

This section of the dissertation aims to break down the results presented in the present study and discuss them by comparing them to the literature.

1. Synaptic Density is Not Affected by the Lack of Alix

Morphological studies⁷ revealed that there is no difference in the synaptic density between wild-type (WT) and Alix knockout (KO) neurons. Firstly, Golgi-Cox staining revealed that the lack of Alix does not affect dendritic spine density in brain slices. Secondly, electron and fluorescence microscopic studies suggested no difference in synaptic density between WT and Alix KO neurons, whether *in situ* or *in vitro*. These results indicate that Alix is not necessary for the formation of synapses.

It was previously demonstrated that the dendritic arborisation is reduced in the cortex from Alix KO mouse brains (Laporte *et al.*, 2017). The same phenomenon was observed in cultured hippocampal neurons at 1 day *in vitro* (DIV) where the length of dendrites and axons were shorter than their counterparts in WT neurons. In the same study, Sholl analysis of hippocampal neurons at 13 DIV showed reduced dendritic arborisation. Therefore, it seems that Alix may have a role in the neurite outgrowth, but not in synaptogenesis.

⁷ Golgi-Cox staining and electron microscopic work have been performed by Marine Laporte and José Martínez-Hernández.

2. Changes in Synaptic Morphology in Alix KO Neurons

A closer examination of electron micrographs exposed differences in synaptic morphology between WT and Alix KO neurons. These include a reduction in the number of synaptic vesicles, an increase in the presynaptic bouton surface area, a reduction in the PSD length and a reduction in the spine head to neck ratio (not shown), all in Alix KO synapses compared to the WT counterparts.

The presynaptic changes will be discussed first. It was mentioned that SV endocytosis serves to regenerate SVs from the presynaptic membrane to replenish the limited pool of SVs and to prevent the expansion of presynaptic membrane. In this regard, the reduction in the number of SVs and increase presynaptic bouton surface area suggests impaired SV endocytosis.

Another possible explanation for the reduction in the number of SVs comes from the capacity of Alix to regulate the actin cytoskeleton. The downregulation of Alix has been shown to cause abnormal organisation of the actin cytoskeleton in different types of cells (Cabezas *et al.*, 2005; Campos *et al.*, 2016). The role of Alix in actin regulation will be discussed in more detail in a later section of the discussion. Actin is known to be abundant in presynaptic boutons and to take part in a number of different processes (see figure 10; Rust and Maritzen, 2015). It was hypothesised that actin has a role in SV pool organisation since actin could be found encompassing the reserve pool of SVs through its interaction with synapsin (Hilfiker *et al.*, 1999). Furthermore, in large synapses such as the Calyx of held, actin filaments stretch from the SVs of the reserve pool to the active zone, to allow the transport of SVs (Sakaba and Neher, 2003). In small hippocampal synapses, actin was shown to regulate neurotransmitter release efficiency by coordinating the positioning of the recycling pool relative to the active zone (Morales *et al.*, 2000). Actin has also recently been shown to be involved in the exchange of SVs between synaptic boutons. A study in hippocampal neurons proposed a role of actin and myosin in SV exchange between synaptic boutons (Gramlich and Klyachko, 2017). The study shows that the 'recently endocytosed' SVs, which are labelled with a fluorescent lipophilic marker, move out of synaptic boutons and migrate to neighbouring boutons. Such SV exchange between synaptic boutons was reduced when the actin cytoskeleton was destabilised or when the ATP/ADP cycle of myosin-V was blocked

by pharmacological interventions. It could be interesting to examine if SV exchange between synaptic boutons is impaired in Alix KO neurons by performing experiments similar to that of the mentioned study.

On the postsynaptic terminal, it was shown that the length of PSD was reduced in Alix KO neurons. This correlates well with the fact that the SV number decreased in Alix KO neurons since the size of PSDs and SV number correlate well (Harris and Stevens, 1989). The spine head-to-neck ratio was also reduced in the Alix KO neurons. These are signs of immature synapses. Our earlier work demonstrates that the ESCRT machinery is needed for the proper development of dendritic spines (Belly *et al.*, 2010). In this study, knockdown of CHMP2B (of the ESCRT-III complex) by RNA interference or the overexpression of mutated forms of the protein leads to abnormal development of dendritic spines. The capacity of CHMP2B to polymerise and form helical structures that constrict the plasma membrane to form tubular protrusions was demonstrated *in vitro* (Lata *et al.*, 2008; Bodon *et al.*, 2011). In addition to CHMP2B, it was suggested that CHMP4B may be involved in the maturation of synapses in a study on *Drosophila* that showed that the mutation of the *shrub* gene (homologous of CHMP4) led to abnormalities in neuronal morphology (Sweeney *et al.*, 2006). Such abnormalities were reverted with the ectopic expression of CHMP4B gene from mice. Taking these studies together, it was then hypothesised that a complex of CHMP2B, CHMP4B and Alix may be necessary for the constraining of the membrane in the neck of dendritic spines (Chassefeyre *et al.*, 2015). This is reminiscent of the role of this tripartite complex in membrane deformation in cytokinesis (Carlton and Martin-serrano, 2007; Henne *et al.*, 2013), membrane repair (Jimenez *et al.*, 2014; Scheffer *et al.*, 2014) and viral budding (Strack *et al.*, 2003; Carlton *et al.*, 2008; Jouvenet *et al.*, 2011). In these processes Alix regulates the recruitment and stability of the protein complexes. Unlike in the cited processes above, where Alix concentrates where it is needed, we were not able to detect Alix recruitment to postsynaptic terminals during stimulation. However, it could be tested whether rescuing the expression of Alix restores the PSD length and spine head to neck ratio. This could be achieved by performing *in vivo* electroporation to rescue the expression of wild-type Alix in the neurons of Alix KO pups. Then by the use of electron microscopy, estimate the PSD length and spine head to neck ratio.

3. Changes in Synaptic Function in Alix KO Neurons

Electrophysiological studies, carried out by Marta Rolland (Grenoble institute des Neurosciences, Grenoble) in collaboration, allowed the study of the synaptic function. We found that the current-voltage curve shifted up slightly, but not significantly, in Alix KO neurons, which implies no change in the basal activity or synaptic connectivity. The experiment, however, determined electrophysiological recordings from a population of neurons and thus, does not provide a conclusion on whether there is a difference at the synaptic level. This can be overcome by performing patch-clamp to obtain the current-voltage curve of individual synapses.

The lack of significant change in the current-voltage curve was supported by the calcium influx assay which reveals that there is no difference in the stimulation-induced uptake of calcium between WT and Alix KO neurons. Again, the experiment takes into account fluorescence changes occurring in a population of cultured neurons. To test whether there are differences in calcium influx at synaptic level, genetically encoded calcium indicators that are specifically expressed in presynaptic boutons could be used (Jackson and Burrone, 2016; Al-Osta et al., 2018). Such indicators comprise a fragment of a SV protein fused to a fluorescent calcium indicator which changes fluorescence depending on concentration of calcium.

Paired-pulse facilitation (PPF) was lower in Alix KO neurons compared to WT neurons. As mentioned in the introduction, excitatory postsynaptic potentials (EPSPs) are measured in response to two presynaptic stimuli in close succession giving rise to paired-pulse ratio. An increase in this ratio, in other words, higher postsynaptic response to the second stimulus compared to the first is termed PPF. A lower PPF was observed in Alix KO hippocampi compared to WT. The present study shows that the total number of SVs is lower in Alix KO synapses compared to WT synapses, and in this sense, it could be postulated that this reduction is accompanied by a concomitant decrease in the number of docked SVs (this has not yet been estimated). In this case, less SVs will be available for fusion in response to the second stimulation in Alix KO synapses, leading to a reduced PPF compared to WT synapses. The results of the present study do not allow us to conclude on this matter since we have not estimated the size of the readily releasable pool, but it could be estimated by the use of

membrane dyes such as FM1-43. FM1-43 is a styryl dye which fluoresces when it incorporates into membranes. When SV recycling is induced in the presence of the dye, it will be incorporated in endocytosed vesicles. Therefore, when excess dye is washed out, the detected fluorescence will be from the FM1-43-containing vesicles. Readily releasable pool in hippocampal synapses can be estimated by stimulating the neurons at 20Hz for 2.5s (which depletes the readily releasable pool) in the presence of FM1-43 (Murthy and Stevens, 1998). As the amount of membrane fused via exocytosis equates the amount of membrane retrieved by endocytosis, the SVs endocytosed immediately after such stimulation represents the readily releasable pool. Therefore, the fluorescence intensity measured after washing off the excess dye corresponds to the readily releasable pool.

Miniature excitatory postsynaptic currents (mEPSCs) occur when SVs fuse without the presence of any stimulation or action potential. The amplitude of mEPSC correlates with neurotransmitter content per vesicle, occurrence of multiple SV fusion, the number of or the type of postsynaptic receptors. No change in the amplitude of mEPSC was observed in Alix KO neurons. This correlates well with the fact that there is no difference in SV size between WT and Alix KO neurons. The frequency of mEPSC correlates with release probability, the number of synaptic vesicles or the number of silent synapses (synapses with NMDAR and without AMPAR). Reduced frequency of mEPSC was observed in Alix KO neurons and this correlates well with the reduced number of SVs in Alix KO neurons.

Electrophysiological studies further revealed that the synapses of Alix KO neurons exhaust more rapidly. It should be noted that synaptic depression occurs within the first few stimulations which is reminiscent of the short-term depression which arises from a slowed release site clearance (Neher, 2010; Hua *et al.*, 2013). In a recent study on cultured hippocampal neurons, it was shown that the impairment of SV endocytosis could lead to a slowed clearance of SV proteins from the release site which hinders further SV fusion, giving rise to short-term depression (Hua *et al.*, 2013). The rapid exhaustion of SVs in Alix KO neurons supports the morphological study which shows reduced number of SVs in Alix KO synapses. Such presynaptic defects could underpin the LTP defect in the Alix KO hippocampus since LTP is induced by high-frequency stimulation. It could be postulated that a rapid depletion of the SVs in response to the LTP-inducing high-frequency stimulation, in

Alix KO synapses, lead to problems in inducing LTP. A patch-clamp experiment should be done to depolarise the pre- and postsynaptic terminals at the same time to induce LTP. In this condition, mild stimulations of the pre- and postsynaptic terminals would allow for the unblocking of NMDARs which is necessary to induce LTP (Lüscher *et al.*, 1999). This protocol is much less sensitive to presynaptic defects since it involves lower frequency stimulation.

4. Recruitment of Alix and Its Partners to Presynaptic Boutons

Western blot⁸ revealed that Alix concentration in cultured neurons increases with time and this increase is in synchrony with increase in PSD95 expression. Interestingly, Alix is found in synaptosomes (membranes enriched in pre- and postsynaptic terminals) in basal conditions (without stimulation). The concentration of Alix in synapses increased when neurons were stimulated with Bic/4AP (bicuculline/4-aminopyridine) stimulation solution, indicating that Alix may have a role in synapses during synaptic activity.

Bicuculline is a competitive antagonist of γ -aminobutyric acid A receptors (GABA_AR), receptors of the major inhibitory neurotransmitter, GABA_A, and 4-aminopyridine is a weak, non-selective blocker of voltage-gated potassium channels. Bicuculline has been used as a tool to study epileptic seizures in rodents since treatment with bicuculline caused an imbalance of excitation and inhibition, leading to excessive action potential discharge, leading to epileptic seizures *in vivo* (Baram and Snead, 1990; Scharfman, 2007). In a separate study, it was shown that indeed the treating hippocampal neurons with bicuculline gave rise to bursts of high frequency action potentials (Hardingham *et al.*, 2002). The frequency of these bursts were increased greatly with the addition of 4-aminopyridine.

This would imply that Alix concentrates in (or recruits to) synapses during intense or high frequency stimulation. Upon quantification, we have found that such recruitment of Alix is to the presynaptic boutons and not to the postsynaptic terminals. Indeed, live imaging revealed that mCherry tagged Alix concentrates in presynaptic boutons that were undergoing synaptic activity, as marked by synaptophysin-pHluorin response. A gradual decrease in the concentration of Alix in presynaptic boutons, back to the basal level, can be observed when the stimulation solution was washed out. This implies that the recruitment of Alix is stimulation-dependent. The recruitment of Alix has not been examined under mild

⁸ Performed by Marine Laporte

or low frequency stimulation. It would be interesting to see if the removal of 4-aminopyridine from the stimulation solution has an impact on the recruitment of Alix since this would reduce the frequency of action potential bursts (Hardingham *et al.*, 2002). Alternatively, low frequency electrical stimulation could be used to see if this leads to the recruitment of Alix to presynaptic boutons.

Interestingly, the pattern of protein recruitment seen in the live imaging videos resemble axonal varicosities observed in damaged axons, as shown in figure 20. A study by Gu *et al.*, 2017, showed that mechanical stress on hippocampal neurons leads to a rapid and reversible formation of axonal varicosities (this is referred to as axon beading). These varicosities were not presynaptic boutons as they were not labelled with presynaptic markers, bassoon and VAMP2. The mechanical stress in this study was induced by the puffing of a pipette (figure 20), which may resemble the environment of the live imaging chamber used for the present study. A perfusion system was used which includes solution flowing in through an inlet at around 2~3ml/minute and a pump connected to the outlet to aspirate excess solution at around the same rate. It is possible that the flow of solution applied mechanical stress on the hippocampal neurons, leading to the formation of varicosities which we may have mistaken for the recruitment of Alix. However, there are two arguments against this notion. First, Alix fluorescence increases in *bona fide* presynaptic boutons that are undergoing synaptic activity as reported by synaptophysin-pHluorin. Second, no axon beading (spots of intense fluorescence) is observed in the axons of neurons expressing mCherry alone (figure 19), indicating that stress-induced axonal varicosities does not form under our experimental conditions. These support the interpretation of this present study that the increase in fluorescence in axons represents protein recruitment to activated presynaptic boutons.

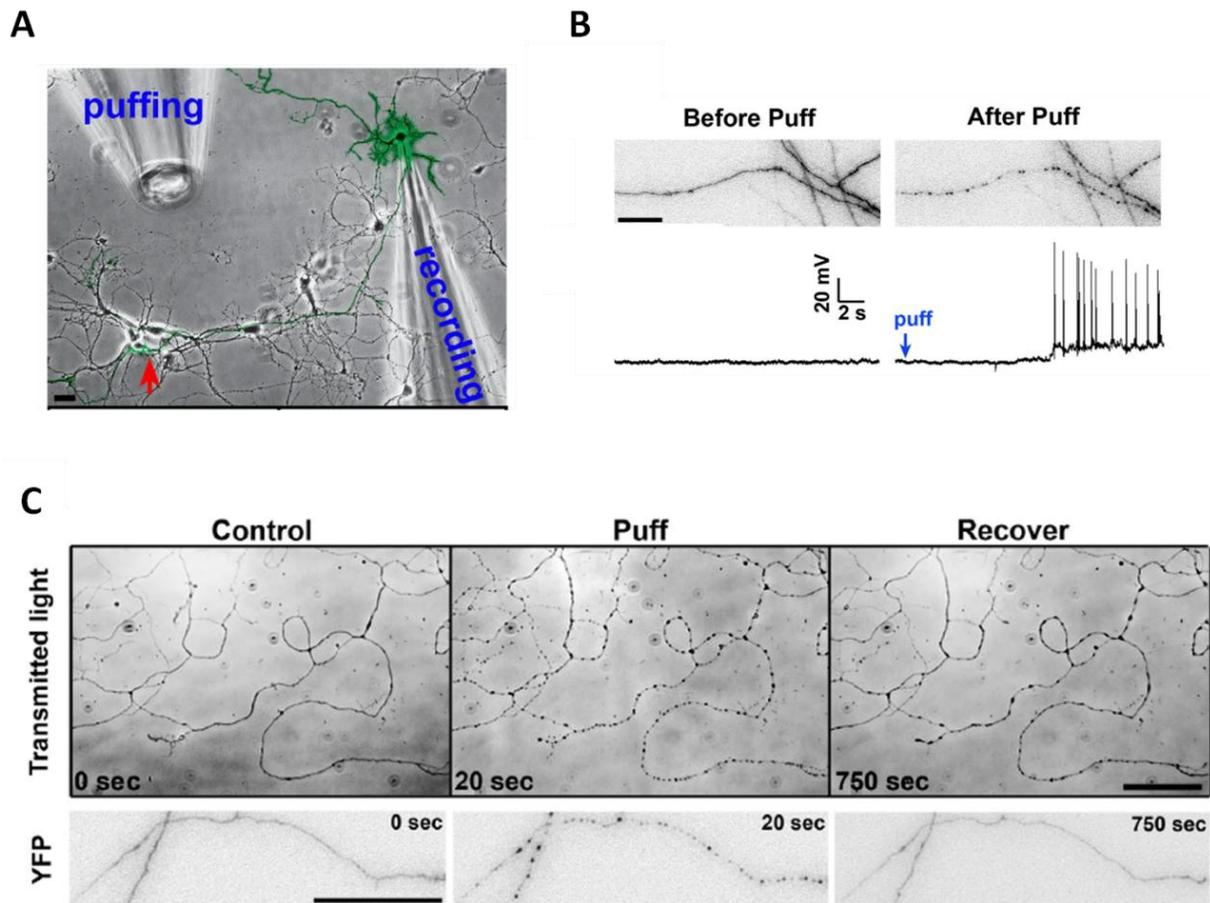


Figure 20: Axon beading induced by mechanical stress

A) A picture depicting the experimental setup. A neuron expressing YFP was patched to record the back-propagating action potentials. Mechanical stress was induced by the puffing of a pipette. **B)** Top panels show axon segments of neurons at 16 days *in vitro* that display axon beading in response to mechanical stress. Bottom panels show the back-propagating action potentials in the same neuron. It is argued that back-propagating action potentials are a way of communicating axonal damage to the soma. **C)** Rapid and reversible axon beading induced by mechanical stress. Adapted from (Gu *et al.*, 2017).

The increase in the abundance of Alix in synaptic regions in response to intense synaptic activity has already been described previously, *in situ*, by our research group (Hemming *et al.*, 2004). The study set out to examine a possible role for Alix in cell death which underlies neurodegeneration induced by kainate in an experimental model of epilepsy, a neurological disorder which is marked by excessive levels of neuronal activity leading to seizures. A widespread low expression of Alix was found in the rat brain and in particular, the neuropil of the CA1 region showed light expression of Alix. Two hours after intraperitoneal

injection of kainate, the expression of Alix increased in various parts of the hippocampus including the neuropil of the CA1 region that corresponded to regions positive for endophilin and synaptophysin. In other words, Alix expression increased in presynaptic boutons. At this time point, increase in c-fos expression indicated kainate-induced synaptic activity, but there was very little change in histology suggesting no cell death. The increase in Alix expression during intense activity dropped back when seizures were suppressed suggesting transient increase, similar to the results of the present study. It was postulated that Alix may be involved in SV endocytosis to maintain synaptic efficiency. The results of the present study support this notion, suggesting that the upregulation of Alix in areas of high neuronal activity (induced by kainate) might be to mediate SV endocytosis. To take the argument further, as the *in vitro* recruitment results of the present study reflect well the observations made previously *in vivo*, it could be considered that role of Alix revealed in the present study represents well the physiological role of Alix. Further study can be done to examine kainate-induced epilepsy in Alix KO mice. In accordance with the results of the present study, we would expect an increase in the presynaptic terminal surface area and perhaps a reduced occurrence of epileptic seizures due to reduced number of synaptic vesicles. To my knowledge, there are no direct studies investigating the significance of ADBE in epilepsy (whether *in vitro* or *in vivo*), therefore Alix KO mice represent a useful tool to this end.

Alix recruitment was completely blocked in cultured neurons that were pre-treated with intracellular calcium chelators EGTA-AM or BAPTA-AM⁹. It was also demonstrated that ADBE was completely blocked in cultured neurons pre-treated with EGTA-AM and BAPTA-AM. This suggests a link between Alix recruitment and ADBE, and implies that both are calcium-dependent.

Synaptophysin-pHluorin response to Bic/4AP stimulation was reduced significantly in the EGTA-AM treated group and even more in the BAPTA-AM treated group (figure 18). For the duration of the 5-minute stimulation, the area under the curve of synaptophysin-pHluorin profiles were significantly reduced in EGTA-AM and almost completely blocked in BAPTA-AM treated groups suggesting significant reduction in or near-complete blockage of SV fusion. It has been published that EGTA, which has a slower on-rate ($k_{on} = 1 \text{ mM}^{-1}\text{ms}^{-1}$), is unable to prevent calcium from triggering exocytosis, but BAPTA, which has a faster on-rate

⁹ AM (acetoxymethyl) will be cleaved off by esterases once inside a cell, leaving just EGTA or BAPTA.

($k_{on} = 100 \text{ mM}^{-1}\text{ms}^{-1}$), is able to bind calcium fast enough to prevent exocytosis (Kits *et al.*, 1999; Morton *et al.*, 2015). It can be envisaged, therefore, that during the prolonged Bic/4AP stimulation, EGTA will act to lower intracellular calcium and thereby reducing SV fusion, whereas BAPTA will compete for calcium at the active zone, leading to a near-complete blockage of SV fusion.

The experiment, which was inspired by Morton *et al.*, 2015, was initially set up in hope of differentiating whether the recruitment of Alix is linked to SV exocytosis or endocytosis, as it still remained that Alix may have a role in SV exocytosis. Morton *et al.*, 2015, show that cultured cerebellar granule neurons that were pre-loaded with FM1-43, released the dye upon stimulation in the presence of intracellular EGTA, but failed to release in the presence of intracellular BAPTA (Morton *et al.*, 2015). In terms of SV endocytosis, both chelators were efficient at blocking ADBE. BAPTA blocked CME whereas EGTA only reduced it, which reflects a tighter coupling of intracellular calcium concentration with ADBE than with CME. Although the concentrations of EGTA-AM and BAPTA-AM used in the present study is the same as those of Morton *et al.*, 2015, there are critical differences. Instead of cerebellar granule neurons, we used hippocampal neurons, and instead of electrical stimulation, we used Bic/4AP stimulation. This means that the intracellular concentration of calcium chelators in our hippocampal cultures may be different from that of the cited study. In addition, the stimulation paradigm used in the present study is subject to the action of calcium chelators whereas the cited study uses an electrical stimulation which will depolarise neurons regardless of the presence of the calcium chelators. In other words, reducing intracellular calcium levels by the introduction of calcium chelators in our experiment will lead to an overall reduction in synaptic activity. It would be interesting to test whether Alix recruitment still occurs when SV fusion is compromised. This could be done by performing a similar recruitment assay, but by depolarising neurons electrically instead of using Bic/4AP stimulation, using cultured neurons incubated with botulinum or tetanus toxins that cleave SNARE proteins to block exocytosis.

There is a tight link between Alix recruitment and calcium. Indeed, Alix recruits to the plasma membrane during membrane repair in a calcium and ALG-2 dependent manner (Scheffer *et al.*, 2014). A similar scenario could be envisaged regarding the recruitment of Alix to the presynaptic terminal since, as mentioned, calcium influx is at the core of synaptic

transmission and SV recycling. ALG-2 is also recruited to presynaptic boutons upon Bic/4AP stimulation, and this recruitment is abolished with a mutation that prevents the interaction of ALG-2 with calcium. An Alix mutant that no longer interacts with ALG-2 failed to recruit to presynaptic boutons suggesting an ALG-2 dependent recruitment of Alix to presynaptic boutons. Taken together, it would appear that synaptic activity-induced calcium influx triggers the recruitment of ALG-2, which in turn recruits Alix to presynaptic boutons. To confirm the results that the recruitment of Alix is ALG-2 dependent, it would be interesting to see if Alix recruitment to presynaptic boutons during intense stimulation still occurs when ALG-2 is knocked-down by using siRNA. It is also possible to infuse peptides that will competitively bind ALG-2-binding region on Alix (La Cour *et al.*, 2018) to see if the recruitment of Alix is dependent on its ability to interact with ALG-2.

Endophilin-A are partners of Alix that also show stimulation-dependent recruitment to presynaptic boutons. The recruitment of endophilin-A is dependent on their ability to interact with Alix, but the recruitment of Alix occurs irrespective of its ability to interact with endophilin. Endophilin-A are a subfamily N-BAR proteins involved in various modes of SV recycling. As mentioned, endophilin-A are implicated in CIE in fibroblasts and this function depends on its ability to interact with Alix (Mercier *et al.*, 2016). The disruption of this interaction leads to impaired CIE. It was proposed that endophilin-A are vital elements of the fast-endophilin-mediated endocytosis (Boucrot *et al.*, 2014), a type of endocytosis that may also be found in synapses (Llobet *et al.*, 2011). Taken together, it seems likely that Alix recruits endophilin-A to presynaptic boutons during intense stimulation to allow endophilin to mediate its role in SV recycling.

The endophilin-A subfamily comprises endophilins-A1, -A2 and A3 that have closely resembling SH3 domains, and all of these isoforms are capable of interacting with the PRD of Alix (Chatellard-Causse *et al.*, 2002). Endophilin-A2 is the isoform examined in the present study which was chosen arbitrarily. In a past study it was shown that endophilin-A2 is strongly expressed in brains and testes of rats, suggesting an important role of this protein in the two organs (Ringstad *et al.*, 2001). Alix also seems to be important in brains and testes as Alix KO mice have smaller brains and testes compared to WT mice (Laporte *et al.*, 2017). Additionally, endophilin-A2 was found to be concentrated in nerve terminals, colocalising with synaptophysin in rat hippocampal neurons (Ringstad *et al.*, 2001). It was also shown

that endophilin-A2 forms complexes with dynamin-1 and synaptojanin-1 suggesting a role in SV endocytosis.

However, it should be noted that endophilin-A1 is the isoform implicated in ADBE since it was among the proteins purified with bulk endosomes (Kokotos *et al.*, 2018). In an earlier study, it was shown that overexpressing a dominant-negative mutant form of endophilin-A1 impairs SV endocytosis at high frequency (40Hz; Kononenko *et al.*, 2014). This could mean that under physiological conditions, Alix may mediate the recruitment of endophilin-A1 to mediate ADBE. Therefore, the possible recruitment of endophilin-A1 should also be examined. Experiments using wild-type and Alix KO neurons transfected with endophilin-A1-mCherry, similar to endophilin-A2 of the present study, could be done to see if endophilin-A1 is indeed recruited in an Alix-dependent manner.

It is shown that Alix recruits to a subset of presynaptic boutons upon intense stimulation. Of all the synapses analysed, 37.0% showed significant increase in the fluorescence intensity of Alix-YFP (similar values were obtained for ALG-2 and endophilin recruitment; table 3). This is interesting as it seems to be in line with the notion that ADBE occurs in a subset of synapses (Clayton and Cousin, 2009a; Nicholson-Fish *et al.*, 2015). A study by Clayton and Cousin, 2009, demonstrated that dextran uptake during intense stimulation was limited to a subset of FM1-43 positive synapses (FM1-43 labels all endocytosed membrane compartments), suggesting that ADBE occurrence is restricted to a subgroup of synapses (Clayton and Cousin, 2009a). In a more recent study, it was suggested that approximately 40% of the synapses analysed in hippocampal cultures exhibited ADBE (Nicholson-Fish *et al.*, 2015). Alternatively, the percentage estimated could simply reflect the heterogeneity of the neuronal cultures in terms of inhibitory synapses (silenced by Bic/4AP stimulation) and excitatory synapses.

5. ADBE Impairment in Alix KO Neurons

The depletion of Alix leads to reduced ADBE as demonstrated using dextran and HRP uptake assays¹⁰, while leaving intact other modes of SV endocytosis that occur in response to low frequency stimulation as demonstrated by the synaptophysin-pHluorin response. The role of Alix in ADBE depends on its ability to interact with ALG-2 and endophilin, but not CHMP4B. The role of the interaction between ESCRT-III and Alix in mediating CIE is not entirely clear. Indeed an Alix mutant which has lost its interaction with CHMP4B does not fully restore the uptake of CTxB (cholera toxin B chain) through CIE (Mercier unpublished). We have not detected such impairment in the present study.

The experiment using pHluorin reporter (synaptophysin-pHluorin) has been performed by Magalie Martineau (Institut interdisciplinaire de Neurosciences, Bordeaux) in collaboration. The fluorescence profiles seem to show no difference between WT and Alix KO hippocampal neurons, but upon analysing the time constant for the fluorescence decay, we have found a reduced time constant in Alix KO neurons. This suggests an increase in the rate of SV endocytosis in Alix KO neurons stimulated at 40Hz. It has been suggested that synaptophysin-pHluorin does not report ADBE (Nicholson-Fish et al., 2015). Therefore, it seems that the impairment of ADBE in Alix KO synapses leads to a compensatory mechanism which increases the rate of Alix-independent SV endocytosis. The mode of SV endocytosis monitored by this reporter might be CME (Nicholson-Fish et al., 2015) or a form of CIE which is formin-dependent (Soykan et al., 2017). One study shows that synaptophysin-pHluorin fluorescence decrease (which indirectly reports endocytosis) is slowed when clathrin heavy chain is downregulated (Nicholson-Fish et al., 2015), while the other study shows that it is unaffected by clathrin heavy chain knockdown (Soykan et al., 2017). The latter study attempted to reconcile the discrepancy by suggesting that the difference derives from the method of transfection used to induce knockdown of clathrin heavy chain. Both calcium phosphate and lipofectamine transfection methods reduce clathrin expression to a similar degree, however, it was hypothesised that the lipofectamine transfection method affects pHluorin profile by slowing the acidification rate (Soykan et al., 2017). Lipofectamine treated neurons displayed reduced mitochondrial activity, which would lead to reduced production

¹⁰ Fiona Hemming and Marine Laporte performed the HRP uptake assay.

of adenosine triphosphate (ATP) and it was argued that this would lead to slower acidification of endocytosed membrane compartments via the ATP-dependent proton pump, H⁺-ATPase. If this argument is true, a similar phenomenon should have been displayed in the control experiments shown in Nicholson-Fish *et al.*, 2015, where neurons were transfected with scrambled shRNA via the lipofectamine-based method.

This brings us to the recent controversies that surround SV recycling mechanisms in hippocampal neurons. It was already mentioned that the significance or even the very existence of CME at hippocampal neurons was challenged by studies that show SV endocytosis in hippocampal neurons occur through clathrin-independent mechanisms at physiological temperatures (Watanabe *et al.*, 2013b, 2014; Delvendahl *et al.*, 2016; Soykan *et al.*, 2017). One such study also showed that at 27°C (low temperature) SV endocytosis occurred as normal even when clathrin heavy chain or AP2 was reduced (Kononenko *et al.*, 2014). In fact, only vesicle budding from endosomes was impaired under such conditions, implying that clathrin is merely necessary for the generation of SV from endosomes. A recent study used a technique to track the exocytic and endocytic events of a single SV to identify three different endocytic pathways (Chanaday and Kavalali, 2018). These three pathways are ultrafast (~150 to 250ms, representing 20% of all endocytic events), fast (~5 to 12s, representing 40% of all endocytic events) and ultraslow (>20s, representing 40% of all endocytic events) pathways that were observed in their relative proportions even at 24°C. This suggests that a form of ultrafast can occur at low temperatures, perhaps without the need of the temperature sensitive actin. It is difficult to reconcile all these studies that appear contradictory. It could be thought that the discrepancies simply arise from the existence of a range of endocytic modes which allow the replacement of one mode should it be compromised. It is also possible that all forms of endocytosis exist in synapses, but each synapse may differ in their capacity to mediate the different forms of endocytosis, depending on the presence of the various endocytic proteins. The results which indicate that ADBE occurs in a subset of synapses support this notion (Clayton and Cousin, 2009a; Nicholson-Fish *et al.*, 2015). Further works unveiling the mechanisms underlying SV recycling is therefore imperative to reconcile the contradictions and to better understand the process in physiological conditions.

6. Possible Mechanisms Underlying the Role of Alix in ADBE

In this section, some of the possible mechanisms of ADBE involving Alix will be discussed by referring to relevant studies.

6.1 Possible Roles of Endophilin

As mentioned in the introduction, BAR domain proteins, such as endophilin, may function by inducing membrane curvature (Frost *et al.*, 2009; Sorre *et al.*, 2012; Shi and Baumgart, 2015). One of the mentioned studies used giant unilamellar vesicles (GUVs) to show that endophilin, if present at high concentrations, induce the formation of tubules on GUVs (Shi and Baumgart, 2015). Interestingly, the study also finds that protein densities at which endophilin acts as membrane inducing modules is attainable in synapses as estimated in isolated synaptosomes, indicating that this function of endophilin may occur under physiological conditions if the protein can be locally concentrated to a sufficient degree. This brings us to the results of the present study, which shows an Alix-dependent increase in the concentration (or density) of endophilin at presynapses during intense stimulation. It could be conceived that such an increase in the density of endophilin in presynapses could lead to BAR-domain induced curvature of the membrane, the tension of which would already be destabilised by the excessive amount of SV fusion to favour deformation. It could be interesting to estimate the density of endophilin in synaptosomes after intense stimulation to see if the increase in the density is sufficient to support this hypothesis. Alternatively endophilin may function as a scaffold protein. The SH3 domain of endophilin interacts with N-WASP which is involved in actin polymerisation (Stanishneva-Konovalova *et al.*, 2016). It seems possible that endophilin may be necessary for the possible role of actin in ADBE (Clayton and Cousin, 2009b; Kononenko *et al.*, 2014).

6.2 Possible Involvement of Actin

Alix has been implicated as a regulator of actin dynamics. In 2005, it was demonstrated that reducing the level of Alix by knockdown leads to the clustering of endosomes to perinuclear regions and a concomitant disruption of the actin cytoskeleton

(Cabezas *et al.*, 2005). Knockdown of Alix led to the abnormal formation of actin aggregates, crescents and rings instead of the normal filaments, and such abnormal forms of actin were found to be enriched in cortactin. It was concluded that Alix is involved in the localisation of endosomes in HeLa cells via its interaction with the cortical actin cytoskeleton. A year later, a study on fibroblasts demonstrated that reducing the level of Alix leads to abnormal cell morphology (Pan *et al.*, 2006). Knockdown of Alix led to reduced expressions of α -actinin, cortactin and F-actin. Furthermore, F-actin to G-actin ratio was reduced strongly suggesting impaired formation of F-actin. Coimmunoprecipitation assay revealed a direct interaction between Alix and various components of the actin cytoskeleton, including α -actinin, cortactin and F-actin. Cortactin activates Arp2/3 to promote actin polymerisation, and α -actinin mediates the bundling of F-actin filaments. It was concluded that reducing the expression of Alix leads to abnormal regulation of the actin cytoskeleton which underlies abnormal cell morphology in Alix knockdown cells. A more recent study reveals a possible role of Alix in the regulation actin in the epithelial cells of the choroid plexus (Campos *et al.*, 2016). Choroid plexus is a single line of cells lining the ventricles of the brain, acting as the blood brain barrier which mediates the separation of blood and cerebrospinal fluid. In the epithelial cells of the choroid plexus of Alix KO brains, fragmentation of actomyosin and aggregation of tight-junction proteins were observed. These led to the loss of cell polarity and promoted the loss of the epithelial layer by increased cell extrusion. It was proposed that such abnormalities in the choroid plexus led to misregulation of the absorption, flow and production of the cerebrospinal fluid, giving rise to hydrocephalus in the Alix KO brain. Interestingly, it was shown that Alix coimmunoprecipitates with F-actin and some tight-junction proteins indicating interaction between these proteins, and strongly indicating the importance of Alix in the regulation of actomyosin and tight junctions.

The possible role of actin in ADBE was already mentioned in the introduction, making the link between various molecules such as syndapin, N-WASP and Arp2/3 (see 'CIE: Activity-Dependent Bulk Endocytosis'). This notion was supported by a study which showed that CIE which occurs in response to high frequency stimulation (40Hz) was slowed by latrunculin A treatment (disrupts actin polymerisation by binding monomeric G-actins; Kononenko *et al.*, 2014). It was also demonstrated that dynamin 1 and endophilin-A1 are required for this mode of SV endocytosis, and the endosome-like vacuoles formed resembled bulk endosomes. This strongly suggests a role for actin and endophilin-A1 in ADBE.

6.3 Possible Mechanism of Action Underlying ADBE Involving Alix

Taking into account the results of the present study and reported functions of Alix and its interacting proteins in membrane trafficking, some possible mechanisms may be speculated (figure 21 illustrates a possible mechanism). Intense stimulation of presynaptic boutons leads to a large amount of SV fusion and a huge increase in intracellular calcium concentration. ALG-2 senses such an increase in calcium and translocates to the presynaptic membrane. The conformational change that occurs when ALG-2 binds calcium allows its interaction with Alix (Maki *et al.*, 2011), leading to the recruitment of Alix to the presynaptic membrane. Alix in turn recruits the N-BAR protein, endophilin-A, increasing the density of endophilin-A at the presynaptic membrane, which leads to the deformation of the presynaptic membrane (Shi and Baumgart, 2015). It is possible that Alix also recruits cortactin, which in turn recruits Arp2/3, to promote actin polymerisation which may be important to mediate membrane invagination. Alternatively, Alix may allow the involvement of actomyosin which could mediate the invagination or constriction of the nascent bulk endosome. SVs are regenerated from bulk endosomes via a clathrin dependent mechanism, and it could be imagined that Alix remains attached to some of these vesicles to allow the transport of them along the actin cytoskeleton for SV exchange between synapses. However, a recent study shows that Alix is not a member of the ADBE proteome, which are proteins purified with bulk endosomes (Kokotos *et al.*, 2018). This suggests that the role of Alix in ADBE may be restricted to the presynaptic membrane to which it may transiently translocate, to recruit other proteins such as endophilin and actin related proteins.

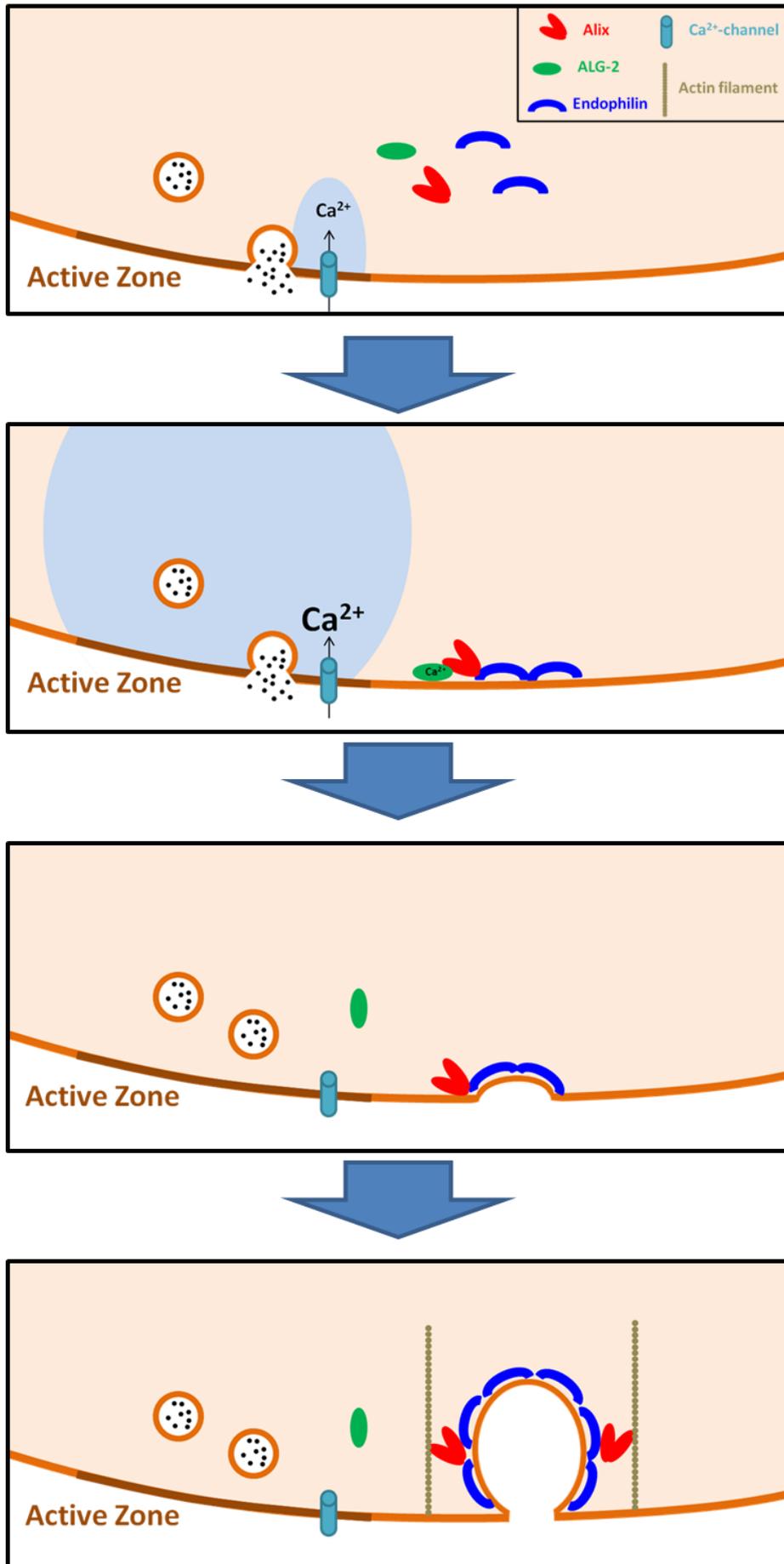


Figure 21: A possible mechanism of action underlying ADBE involving Alix (↑)

Action potentials arriving at the presynaptic terminal trigger the influx of calcium through voltage-gated calcium channels. Microdomain of calcium generated triggers to the fusion of SVs with the presynaptic membrane. Prolonged stimulation leads to an accumulation of calcium, generating larger microdomains of calcium. This triggers the calcium-dependent recruitment of ALG-2, Alix and endophilin to the presynaptic membrane. Increase in the density of endophilin might bring about membrane deformation. The interaction between Alix and actin may be necessary for membrane invagination.

7. General Conclusion

The present study demonstrates the importance of Alix in presynaptic boutons of the central nervous system for SV recycling during intense synaptic activity. This study is the first to show that ALG-2, Alix and endophilin recruit to presynaptic boutons during intense stimulation. Alix depletion led to the impairment of ADBE at synapses demonstrating that Alix is crucial for ADBE. This function of Alix was shown to be dependent on its ability to interact with ALG-2 and endophilin, but not CHMP4B. The underlying molecular mechanism was only inferred from the literature and remains to be explored.

Despite decades of research on the subject, the molecular underpinnings of SV recycling still remains to be explored. This study adds to the growing knowledge of the subject and opens new perspectives.

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Résumé

La neurotransmission nécessite la fusion de vésicules synaptiques (VS) avec la membrane pré-synaptique pour libérer les neurotransmetteurs qui vont activer les récepteurs du neurone post-synaptique. Le nombre de VS dans le bouton pré-synaptique est limité, ce qui nécessite des mécanismes efficaces pour régénérer ces vésicules. Parmi ces mécanismes, il existe l'endocytose dépendante de la clathrine, l'endocytose ultra-rapide et l'endocytose de masse (ADBE), ces deux dernières étant indépendantes de la clathrine. Dans cette étude, il est montré que l'absence d'Alix (ALG-2 interacting protein-X) entraîne des modifications morphologiques et fonctionnelles des synapses qui pourraient être la conséquence d'un recyclage défectueux des VS. Nous avons récemment montré que Alix et l'un de ses partenaires, l'endophiline-A, sont essentiels pour l'endocytose indépendante de la clathrine dans les fibroblastes, ce qui nous amène à penser que Alix pourrait jouer le même rôle dans le bouton pré-synaptique. Au cours de ce travail, j'ai montré que Alix se concentre au niveau des boutons pré-synaptiques après une stimulation à haute fréquence des neurones. En utilisant des formes mutantes d'Alix, j'ai montré que le recrutement d'Alix à la membrane pré-synaptique était dépendant de son interaction avec ALG-2 (protéine de liaison au calcium). De même la relocalisation de l'endophiline-A à la synapse est dépendante de son interaction avec Alix. Par ailleurs, l'absence d'Alix dans les neurones conduit à une altération de l'ADBE, qui peut être restaurée par l'expression d'Alix, mais pas avec ses mutants incapables d'interagir avec ALG-2 ou l'endophiline-A.

Les résultats de cette étude démontrent qu'Alix est important pour l'endocytose indépendante de l'ADBE dans les synapses, processus indispensable au recyclage des vésicules synaptiques au cours d'une activité neuronale normale.

Abstract

Neurotransmission involves the fusion of synaptic vesicles (SVs) to the presynaptic membrane to release neurotransmitters that will bind receptors on the postsynaptic neuron. The number of SVs in central nerve terminals is limited, necessitating a set of reliable mechanisms to regenerate SVs. Among such mechanisms are clathrin-mediated endocytosis, ultrafast endocytosis and activity-dependent bulk endocytosis (ADBE), where the latter two are clathrin-independent. The present study reveals that the depletion of Alix (ALG-2 interacting protein-X) leads to morphological and functional changes of synapses that indicate defective SV recycling. Our recent finding that Alix and its major partner, endophilin-A, is crucial for clathrin-independent endocytosis in fibroblasts, led us to hypothesize that Alix may have such a role at the presynapse. The present study has allowed us to demonstrate that Alix concentrates at presynaptic boutons during intense stimulation. Using mutant forms of Alix, we demonstrate that this recruitment of Alix is depends on the ability of Alix to interact with the calcium binding protein, ALG-2. Endophilin-A is also recruited to presynaptic boutons and this recruitment is dependent on its capacity to interact with Alix. It is then revealed that the lack of Alix in neurons leads to impaired ADBE, suggesting the importance of Alix in ADBE. Such a role of Alix depends on its ability to interact with ALG-2 and endophilin since the impairment of ADBE was reversed upon rescuing the expression of wild-type Alix, but not with Alix mutants unable interact with ALG-2 or endophilin-A.

The findings of this study demonstrate that Alix is important for ADBE at synapses, a process necessary for the recycling of synaptic vesicles during normal neuronal activity.