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Review

Coupling exo- and endocytosis: An essential role for PIP_2 at the synapse ☆

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ABSTRACT

Chemical synapses are specialist points of contact between two neurons, where information transfer takes place. Communication occurs through the release of neurotransmitter substances from small synaptic vesicles in the presynaptic terminal, which fuse with the presynaptic plasma membrane in response to neuronal stimulation. However, as neurons in the central nervous system typically only possess ~200 vesicles, high levels of release would quickly lead to a depletion in the number of vesicles, as well as leading to an increase in the area of the presynaptic plasma membrane (and possible misalignment with postsynaptic structures). Hence, synaptic vesicle fusion is tightly coupled to a local recycling of synaptic vesicles. For a long time, however, the exact molecular mechanisms coupling fusion and subsequent recycling remained unclear. Recent work now indicates a unique role for the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2), acting together with the vesicular protein synaptotagmin, in coupling these two processes. In this work, we review the evidence for such a mechanism and discuss both the possible advantages and disadvantages for vesicle recycling (and hence signal transduction) in the nervous system. This article is part of a Special Issue entitled Lipids and Vesicular Transport.

1. Introduction

1.1. Synaptic vesicle trafficking — an overview

The chemical synapse is the major structure where information is processed and transferred between neurons and their target cells. At the heart of this process is the synaptic vesicle — a small membranous organelle found in the presynaptic terminal, which contains the neurotransmitters that are the basis of neuronal communication. When the presynaptic terminal is depolarized by an action potential, synaptic vesicles fuse with the plasma membrane (exocytosis). Released neurotransmitters then diffuse across the synaptic cleft and attach to receptors on the postsynaptic neuron; depending on the type of neurotransmitter released (excitatory or inhibitory) this may lead to depolarization or hyperpolarization of the postsynaptic neuron, respectively.

A presynaptic terminal in the central nervous system typically contains 200 synaptic vesicles [1], which can be rapidly depleted under conditions of intense stimulation. Given the distance of the presynaptic terminal from the neuronal cell body, replacement of synaptic vesicles by *de novo* synthesis and transport would be too slow to sustain continuous neuronal activity [2]. In addition, unregulated fusion would lead to a dangerous increase in the area of the presynaptic plasma membrane (and possible misalignment with postsynaptic structures). Hence, synaptic vesicle membrane is retrieved (endocytosis), and used to reform synaptic vesicles that are used for subsequent rounds of fusion (see Fig. 1).

Major progress has now been made in understanding the molecular mechanisms underlying exo- and endocytosis. Surprisingly, many of the proteins involved in these processes were found to be part of much larger protein superfamilies that also function in either constitutive fusion of trafficking vesicles or constitutive internalization of receptors [3,4]. However, where synaptic vesicle trafficking differs from constitutive trafficking is that neuronal exo- and endocytosis is a strictly compensatory process — meaning that there must be a cross-talk between the processes that not only matches the number of exocytosed vesicles to those recovered by endocytosis, but also makes sure the vesicles are of the correct size and composition (including the types and copy numbers of proteins).

Two major models have been proposed for how the processes are coupled.

Abbreviations: AP, Adaptor protein; Arf, ADP-ribosylation factor; CALM, clathrin assembly lymphoid myeloid leukemia protein; CAPS, Calcium activated protein for secretion; Cdk, Cyclin-dependent kinase; DAG, Diacylglycerol; EMS, Ethyl methanesulfonate; FCHo1/2, F-BAR domain-containing Fer/Cip4 homology domain-only proteins 1 and 2; GFP, Green fluorescent protein; GST, glutathione S-transferase; HIP, Huntingtin interacting protein; IP_3, Inositol trisphosphate; MARCM, Mosaic analysis with a repressible cell marker; NMJ, Neuromuscular junction; PH, Pleckstrin homology; PIP_2, Phosphatidylinositol 4,5-bisphosphate; PKC, Protein kinase C; PLC, Phospholipase C; PLD, Phospholipase D; PS, Phosphatidylserine; PX, Phox; RIM, Rab3 interacting molecule; SCAMP, Secretory carrier membrane protein; SHD, Stonin homology domain; SNARE, Soluble NSF attachment protein receptor; VAMP, Vesicle associated membrane protein; μHD, mu-homology domain

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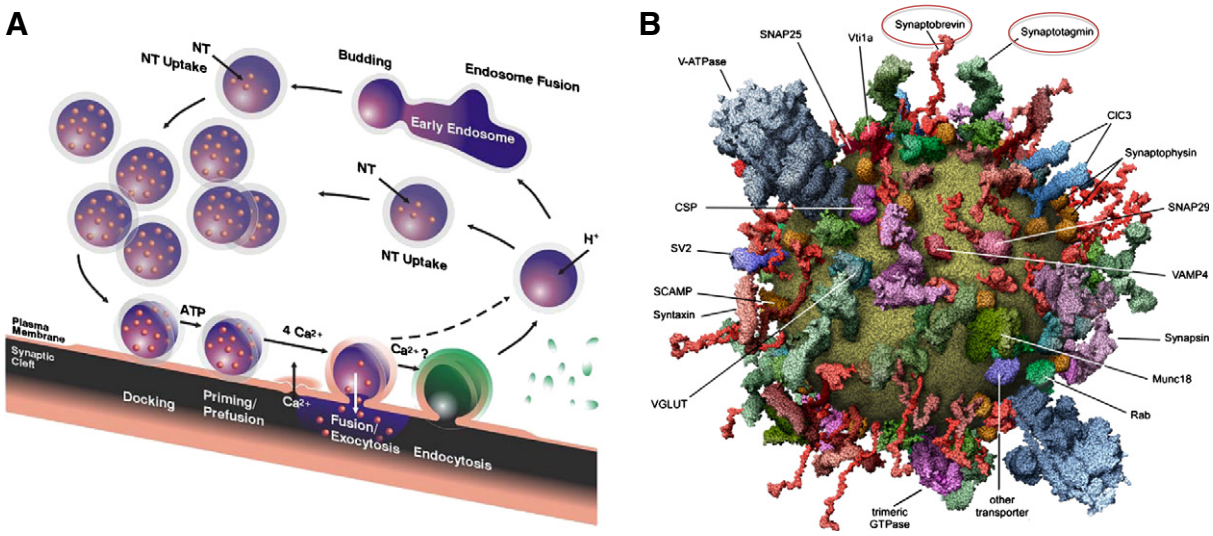


Fig. 1. The synaptic vesicle cycle. (A) Synaptic vesicles are locally recycled in the presynaptic terminal [2,120]. Synaptic vesicles are trafficked to specialist release sites on the plasma membrane - active zones [238]. After docking to release sites, vesicles undergo a complex set of reactions that renders them fusion competent — priming [239]. The arrival of an action potential in the presynaptic terminal leads to the opening of voltage-gated Ca^{2+} channels in the plasma membrane and the subsequent influx of Ca^{2+} — leading to exocytosis [240]. After fusion, vesicle membrane is recovered by endocytosis — a process that may or may not involve clathrin (green) [129]. It is unclear whether endocytosis takes place at the active zone, or occurs at peri-active zones that physically separate the sites of vesicle fusion and retrieval [36,241]. The vesicle lumen is then rapidly acidified by the action of a proton pump [242], which drives the uptake of neurotransmitter into the vesicle [243,244]. It still remains unclear whether recycling vesicles pass through an obligate sorting intermediate (early endosome) that acts as a quality control mechanism, removing damaged vesicle components and allowing their replacement [125–127]. (B) A molecular model of an average synaptic vesicle isolated from rat brain. The model is based on space-filling models of all macromolecules at near atomic scale. As expected, the various trafficking proteins that are known to coordinate key steps in the synaptic vesicle cycle dominate the vesicle surface. All vesicles are thought to share a common set of these proteins, although isoform specificity may vary among synapses. Of particular relevance here are the proteins synaptobrevin and synaptotagmin (highlighted by red rings), which play crucial roles in both exo- and endocytosis (see main text). An average vesicle is thought to contain 70 copies of synaptobrevin and 15 copies of synaptotagmin. Proteins are shown randomly distributed over the vesicle surface, but could also be present in clusters [166,245].
Panel B taken from [52].

The first model treats exocytosis and endocytosis as two mechanistically distinct processes — requiring separate triggers. An obvious candidate for such a trigger is Ca^{2+}. Exocytosis is strictly dependent on high levels of local Ca^{2+}, which are generated at release sites due to an influx through voltage gated Ca^{2+} channels which open during an action potential. Ca^{2+} has also been proposed to positively regulate endocytosis [5–7], as some of the key endocytic proteins are known to be dephosphorylated by the Ca^{2+} activated phosphatase calcineurin [8]. However, whether Ca^{2} acts as a universal trigger remains unclear, as several studies report an actual inhibition of endocytosis by Ca^{2+} [9–11]. There remains the possibility, however, that these discrepancies arise from the use of different synaptic preparations and differing stimulus levels which may invoke different recycling mechanisms [12–14].

The second model envisages that the fusion of the vesicle with the plasma membrane causes a detectable change in the membrane structure – possibly deposition of synaptic vesicle proteins into the membrane – that acts as a signal for membrane to be endocytosed [15].

Interestingly, these two models may not be mutually exclusive: different aspects of exo-endocytic coupling (such as the initiation event and extent of coupling) may be mediated by different mechanisms, such as the local cytoplasmic Ca^{2+} concentration.

Kinetically, the coupling between exo- and endocytosis is fast — with evidence that endocytosis can actually begin immediately after exocytosis [16,17]. Thus, there have been severe technical difficulties to exploring the coupling mechanism — and physically isolating the individual processes. Traditional biochemical/cell biological methods, such as the addition of small molecular weight compounds [18], or the overexpression of dominant negative proteins to interfere with endocytosis [19], take in the order of minutes to hours to act and, together with the high concentrations typically used, can produce many "off-target" effects [20].

To date the "cleanest" approaches have involved the genetic manipulation of exo- and endocytosis. The vesicular Ca^{2+} sensor synaptotagmin has long been recognized as an essential component of the exocytic machinery in neurons [21]. However, recent work using chronic genetic ablation of synaptotagmin in mouse [22], and acute inactivation of the protein in *Drosophila melanogaster* [23], has indicated that not only is this protein involved in neurotransmitter release but it also has a potential role in endocytosis.

Given that exo- and endocytosis are mechanistically very different (see Section 3), how does a single protein like synaptotagmin work to couple them? Is synaptotagmin the principal player? Has our ability to selectively manipulate proteins at the synapse, but not lipids, obscured 'the hub of the wheel'? Is synaptotagmin the focal point of the coupling process, or should our attention actually be diverted elsewhere?

In this review, we will argue that the plasma membrane lipid PIP_2 forms the 'hub' of this essential coupling process, with synaptotagmin acting as an effector protein. We base our case on three main arguments. First, the unique properties of PIP_2 make it an ideal molecule to locally recruit the machinery needed for both exo- and endocytosis [24,25]. Second, there is already substantial biochemical and cell biological evidence that the plasma membrane lipid PIP_2 is actively involved in exocytosis, primarily through its interactions with synaptotagmin [21]. Third, there is substantial evidence for a role of PIP_2 in recruiting essential endocytic proteins following fusion [4], including some which bind synaptotagmin [26]. Finally, we will attempt to illustrate not only the benefits but also the disadvantages of employing such a system.

2. Phosphatidylinositol 4,5-bisphosphate at the presynaptic terminal

2.1. Structure, localization and synthesis

PIP_2 is a member of the phosphoinositide family of membrane lipids, which all share the same basic structure; a long fatty-acyl

chain (of varying length and saturation [27]) embedded in the membrane *monolayer*, and linked to a myo-inositol headgroup via a glycerol backbone. Importantly, reversible phosphorylation of the myo-inositol ring at the 3′, 4′ and 5′ OH positions can generate up to 7 stereospecific isoforms of phosphoinositide [28,29]. PIP_2 is, of course, phosphorylated at the 4′ and 5′ positions (Fig. 2A).

At equilibrium, the total percentage of PIP_2 in neuronal membranes is actually very low. Bulk phosphoinositide profiling of whole brain tissue using electrospray ionization mass spectrometry showed PIP_2 to comprise ~1% of the total lipid [27], which is in good agreement with metabolic labeling/HPLC experiments in cultured cortical neurons [30]. These studies should be considered alongside an elegant set of experiments in which the PIP_2 binding pleckstrin homology (PH) domain of phospholipase C was fused to green fluorescent protein (PLC-GFP). Using this approach, PIP_2 was found localized predominantly to the cytosolic face of the plasma membrane in cultured neurons under resting conditions (Fig. 2B), consistent with antibody labeling [31]. However, staining was found distributed throughout the cell body and neuronal processes. Together, these studies are actually suggestive of rather low concentrations of PIP_2 distributed throughout neuronal membranes. This begs the question of how such low local concentrations of lipid could act as an exo-endocytic hub? A recurrent theme in modern membrane biology is the organization of membrane lipids into preassembled supra-molecular structures (or "clusters") that represent hot-spots for individual biological processes [32]. At least two lines of evidence now point to the fact that PIP_2 is, in fact, highly concentrated at release sites. First, neuronal stimulation leads to an increase in the amount of PLC-GFP in individual synaptic terminals [31], consistent with the burst of PIP_2 synthesis measured biochemically (see below). Second, studies of pure plasma membrane sheets derived from PC12 secretory cells and stained with PLC-GFP and PIP_2 antibodies, suggest the lipid is organized into microdomains at the sites of docked secretory vesicles [33,34]. Importantly, a recent study, which used super-resolution imaging techniques to circumvent the diffraction limit of normal fluorescence microscopy, not only confirmed many of these findings, but also revealed that PIP_2 clusters on membrane sheets are much smaller than anticipated, with an average diameter of only 73 nm [35]. The authors then went on to calculate the surface density of PIP_2 in these clusters based on reference to PIP_2 staining in artificial supported bilayers. Importantly, the authors claim that PIP_2 is the dominant lipid at the center of these domains, comprising a fair higher percentage of the total than previous thought, perhaps even approaching 100% of the total lipid. These experiments should now be repeated with cultured neurons, in combination with super-resolution imaging [36], to investigate the possibility of micro-patterning in the neuronal plasma membrane.

How are these local domains of PIP_2 formed? At the synapse, it is thought that the primary route for PIP_2 synthesis is via phosphorylation of the precursor lipid phosphatidylinositol 4-phosphate by

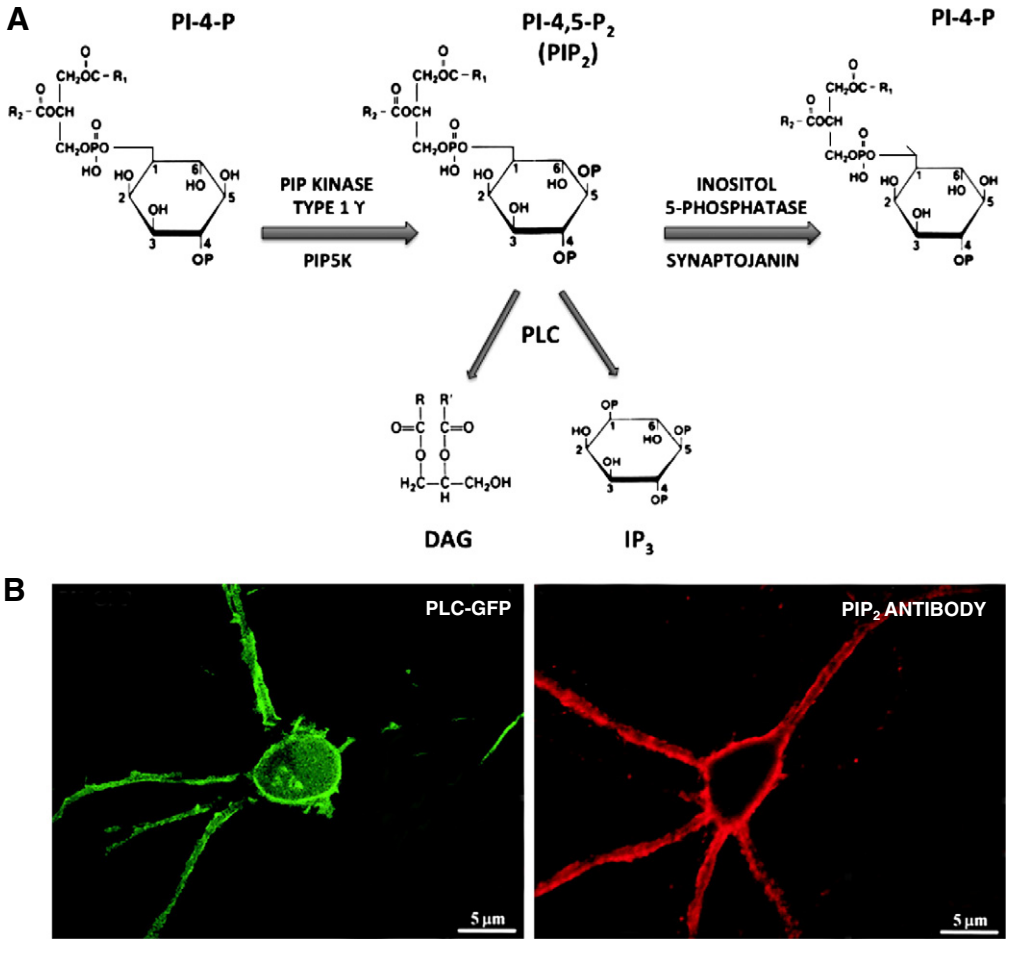


Fig. 2. PIP_2 at the synapse. (A) Principal pathways of PIP_2 synthesis and degradation at the synapse [30,38]. Lipids are labeled to clearly indicate the position of the phosphate group on the myo-inositol ring, and how this varies at various stages of the phosphoinositide cycle. As well as being an important signaling molecule in its own right, PIP_2 can act "indirectly" to modulate synaptic vesicle trafficking; with phospholipase C catalyzed hydrolysis producing the second messenger diacylglycerol (DAG) and inositol trisphosphate (IP_3), both of which are also functionally active at the presynapse [246–249]. (B) In cultured hippocampal neurons, PIP_2 is localized to the cytosolic face of the plasma membrane — as revealed by overexpression of the lipid binding pleckstrin homology (PH) domain of phospholipase C fused to green fluorescent protein (PLC-GFP) or antibody labeling. Image taken from [31].

members of the type 1 phosphatidylinositol phosphate kinase family (PIPK) [37]. In particular, the p90 isoform of PIPK1γ (PIPK1γ-p90) is concentrated at synapses [38,39], and is thought to play the dominant role in PIP_2 synthesis in neurons. Genetic experiments in mice have shown that PIP kinase type 1γ is both necessary and sufficient to support life to adulthood [40], as ablation of this enzyme leads to early lethality. Although the reduction of total PIP_2 content at the synapse is only 40%, such a reduction is presumably lethal because of the profound impairments in neurotransmitter release that result [41], although, as with all constitutive gene "knock-outs", it is impossible to rule out subtle developmental effects in the nervous system. Interestingly, these effects cannot be overcome by the actions of either PIPK1α or PIPK1β, which are actually thought to play overlapping, albeit minor roles, in prenatal and postnatal development [40]. Such a finding is consistent with local and metabolically distinct pools of PIP_2 in neurons (and see below). Crucial to this concept is the finding that PIPK1γ-p90 interacts with the protein talin at synapses, and disruption of this interaction severely perturbs synaptic vesicle trafficking [42]. In unstimulated synapses, this interaction is prevented by phosphorylation of serine 650 (S650) in the talin-binding site of PIPK1γ-p90 by cyclin-dependent kinase 5 (Cdk5). Activation of the Ca^{2+}-dependent phosphatase calcineurin leads to dephosphorylation of S650, allowing the interaction with talin and presumably local PIP_2 synthesis.

Phase locking the activity of PIPK1γ-p90 to Ca^{2+} influx during the action potential would explain why neuronal activity triggers a burst of PIP_2 synthesis [43]. Tying the amount of PIP_2 production directly to the degree of stimulation received by a neuron would also be a simple and effective way of manipulating the dynamic range of the release system [43], and there is already evidence that the size of the rapidly releasable pool depends on PIP_2 levels [33].

In addition, there is strong evidence that PIPK1γ is also stimulated by the small GTPase ADP-ribosylation factor 6 (Arf6) [44,45], which is enriched in presynaptic terminals and localizes to the plasma membrane when activated [46]. Arf6 regulates exocytosis in neuroendocrine cells [47], and it is likely that a similar mechanism exists at neuronal synapses. Any such Arf dependent activation of PIPK1 would presumably also overlap with other Arf-dependent effector pathways, such as the Arf-mediated stimulation of phospholipase D (PLD). PLD is responsible for the hydrolysis of phosphatidylcholine to phosphatidic acid, which is a potent stimulator of PIPK1γ-p90 [48,49] and also regulates neurotransmitter release at synapses [50].

Taken together, it seems likely that there are multiple pathways governing PIP_2 production and synaptic vesicle trafficking at the synapse. However, the relative contributions of these individual pathways (and possible synergism) remain to be fully investigated, but will no doubt form an important part of our future understanding of neurotransmitter release.

In contrast, the origin of phosphatidylinositol 4-phosphate is much more contentious. It appears that a phosphatidylinositol 4-phosphate kinase (PI 4-kinase) is bound to the membrane (of at least a fraction) of synaptic vesicles [51,52]. However, it remains to be established whether the phosphatidylinositol 4-phosphate generated by this vesicle-associated kinase represents the precursor for the PIP_2 needed for exocytosis. In principal, plasma membrane bound PIPK1γ-p90 could phosphorylate phosphatidylinositol 4-phosphate in the synaptic vesicle in a "trans" configuration after synaptic vesicles have docked at the plasma membrane (see Fig. 1A). It is possible that such a reaction might underlie vesicle "priming". This is an attractive possibility as it implies a specific PIP_2 generating system nested within the synaptic vesicle cycle [43]. Alternatively, another PI 4-kinase on the plasma membrane may be used. At present, it is unclear which of these mechanisms predominates at the synapse.

Given the importance of PIP_2 in neuronal function, it is essential that its levels are strictly regulated. As is the case in most biological processes, the level of PIP_2 produced by kinase activity in neurons is not static, even under conditions of low neuronal activity. PIP_2 is known to be dephosphorylated back to phosphatidylinositol 4-phosphate [43], implying a cycle of phosphorylation-dephosphorylation in the terminal. Such a cycle may at first sight seem futile, but it is presumably a well controllable and versatile mechanism to allow rapid changes in effective PIP_2 levels, as neuronal activity triggers a burst of PIP_2 synthesis [43]. The main phosphatase in neurons is the inositol-5-phosphatase synaptojanin 1 (see Fig. 2A). In particular, the 145 kDa isoform of synaptojanin is highly expressed in the brain — and is responsible for terminating the PIP_2 signal following endocytosis (see Section 5). Additionally, PIP_2 is also known to be a substrate for phospholipase C (PLC) in the presynaptic terminal [53], an important second messenger system in exocytosis (see Fig. 2A and Table 1). PIP_2 may also be "inactivated" by phosphatidylinositol 3-kinases (not shown) [54].

2.2. Properties of PIP_2 with relevance for synaptic vesicle trafficking

PIP_2 displays various properties that make it an ideal lipid mediator in synaptic vesicle trafficking. First, PIP_2 is embedded in the inner leaflet of the plasma membrane, which is the site of exo- and endocytosis. This effectively reduces the dimensionality of the system, producing a local concentration effect, which is important in the recruitment of cytosolic factors to the membrane (see Section 3.2). Although the orientation of the headgroup with respect to the membrane remains unknown, the large size implies that it may protrude further into the aqueous phase than other phospholipids facilitating interactions [24,55,56]; a second alternative mode of interaction, in which proteins penetrate the membrane surface and interact hydrophobically with the membrane core, has also been reported [43,57]. Both modes of interaction are important at the synapse, and may play an important role in distinguishing mechanistically between exo- and endocytosis. Second, the specific phosphorylation of the myo-inositol headgroup imparts both charge and conformational specificity. Depending on factors such as the local pH, PIP_2 has a net negative charge of between −3 to −5 [24], allowing non-selective electrostatic binding to PIP_2 [58–61]. In addition, the stereospecificity of the phosphorylation sites also imparts a degree of selectivity for specific binding domains [62–64]. Regardless of the manner of binding, however, many proteins also contain additional binding sites for membrane proteins, and an interaction of sufficient affinity only occurs when both binding sites are engaged — effectively creating a coincidence detection system at the synapse, which imparts an extra level of specificity to the system (see Section 3.2) [43]. Finally, the rapid phosphorylation or dephosphorylation of the inositol ring produces "stage-specific" markers, which provide a convenient way to ensure vectorial transport of vesicles through the trafficking cycle [30,65].

3. PIP_2 and synaptotagmin: an essential role in coupling exo- and endocytosis

The processes of exo- and endocytosis place different demands on the synaptic machinery. To fuse a small, highly curved synaptic vesicle with the flat plasma membrane involves a different set of physical constraints than the reformation of a vesicle. Therefore, it is unsurprising that the processes use very different molecular machineries. However, it appears that there is at least one common link between the processes — PIP_2 and synaptotagmin. Work from many laboratories has provided strong evidence that synaptotagmin has functions in both exocytosis and endocytosis. To understand how PIP_2 and synaptotagmin could couple these two events, we will first review the evidence for their roles in the individual processes (this section). In the light of this information, we will then introduce the potential for "coupling" between the processes, and discuss some ideas regarding this process and its potential benefits for neurons (Section 4).

Table 1
PIP_2 binding proteins with a known presynaptic function.

Protein	PIP_2 binding domain	Function	References
Synaptotagmin	C2	• Vesicular Ca^{2+} sensor • Putative molecular link between exo- and endocytosis	[15,82,251]
Doc2	C2	• Putative Ca^{2+} sensor	[252–254]
Rabphilin 3A	C2	• Rab3 effector protein, which acts as a priming factor for synaptic vesicles	[255]
Calcium activated protein for secretion (CAPS)	C2 and PH[a]	• Priming factor for synaptic vesicles	[34,256]
Mint	Phosphotyrosine binding domain	• Control of vesicle docking at active zones by SNARE protein regulation. • Structural organization of active zone by neurexin and Ca^{2+} channel binding	[257–259]
Piccolo/Aczonin	C2	• Scaffolding protein[b]	[110,260]
Rab3 interacting molecule (Rim)	C2	• Scaffolding protein[b]	[110,261]
Type II Phosphoinositide 3 kinase-C2α	PX domain. Also contains a C2 domain.	• Production of 3′ phosphoinositides stimulates exocytosis	[262,263]
Secretory carrier membrane protein (SCAMP) 2	Electrostatics — basic/ hydrophobic amino acids	• Control of fusion pore formation during exocytosis	[264]
Syntaxin 1	Electrostatics — basic amino acids	• SNARE protein	[35,78]
Voltage gated K^+ channels (KCNQ)[c]	Electrostatics — basic amino acids	• Action potential generation	[265,266]
High voltage activated Ca^{2+} channels (L, N and P/Q type)[c]	Electrostatics — basic amino acids?	• Ca^{2+} influx during exocytosis	[266,267]
Phospholipase D (PLD)	PH and basic amino acids	• Hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline - PA acts to stimulate PIP5Kγ - PA induces negative membrane curvature, that may influence exo- and endocytosis	[44,268,269]
Munc13	C1–DAG binding[d]	Priming factor for synaptic vesicles	[246,270,271]
Adaptor protein (AP) subunits α, β_2 and μ_2	Electrostatics — basic amino acids	• Central endocytic hub - Linking plasma membrane cargos to clathrin and endocytic accessory proteins	[4,132]
Epsin 1	ENTH	• Cargo-specific adaptor protein • Involved in membrane remodeling/bending	[4,272,273]
Dynamins 1, 2 and 3[e]	PH	• Membrane scission during final stages of endocytosis	[274,275]
AP180 (and CALM)	ANTH	• Binds AP2 and clathrin - Thought to regulate synaptic vesicle size	[4,276,277]
Huntingtin-interacting protein (HIP) 1	ANTH	• Binds AP2, clathrin and actin	[4,278–280]
ARF6	Electrostatics — basic amino acids	• Stimulation of PIPK1γ - Increase in PIP_2 leads to enhanced AP2 recruitment • Stimulation of PLD (see above)	[281–283]

References are given as sources of domain identification and putative functions.
Proteins with an indirect role in endocytosis via actin modification are not listed.
Most proteins are found in both vertebrates (mouse) and invertebrates (*Drosophila*), with the exceptions of Doc2 and Piccolo, which are only found in mouse.
[a] The use of multiple binding domains is commonly used to stabilize a protein at the membrane, and can also be used to increase specificity due to the element of coincidence detection required to successfully bind specific lipids via both sites.
[b] Scaffolding proteins are large multi-domain proteins present at the active zone, which are thought to play a number of important roles in the presynaptic terminal. A comprehensive overview of their functions is given in the indicated review.
[c] No evidence to date exists for PIP_2 modulation of voltage gated Na^+ channels.
[d] Although Munc13 contains C2 domains, most interest has centered on its activation by DAG — formed by PLC hydrolysis of PIP_2.
[e] Dynamin 1 is found specifically in neurons; dynamin 2 is ubiquitous; dynamin 3 is expressed predominantly in testes and also in lung and neurons [284].

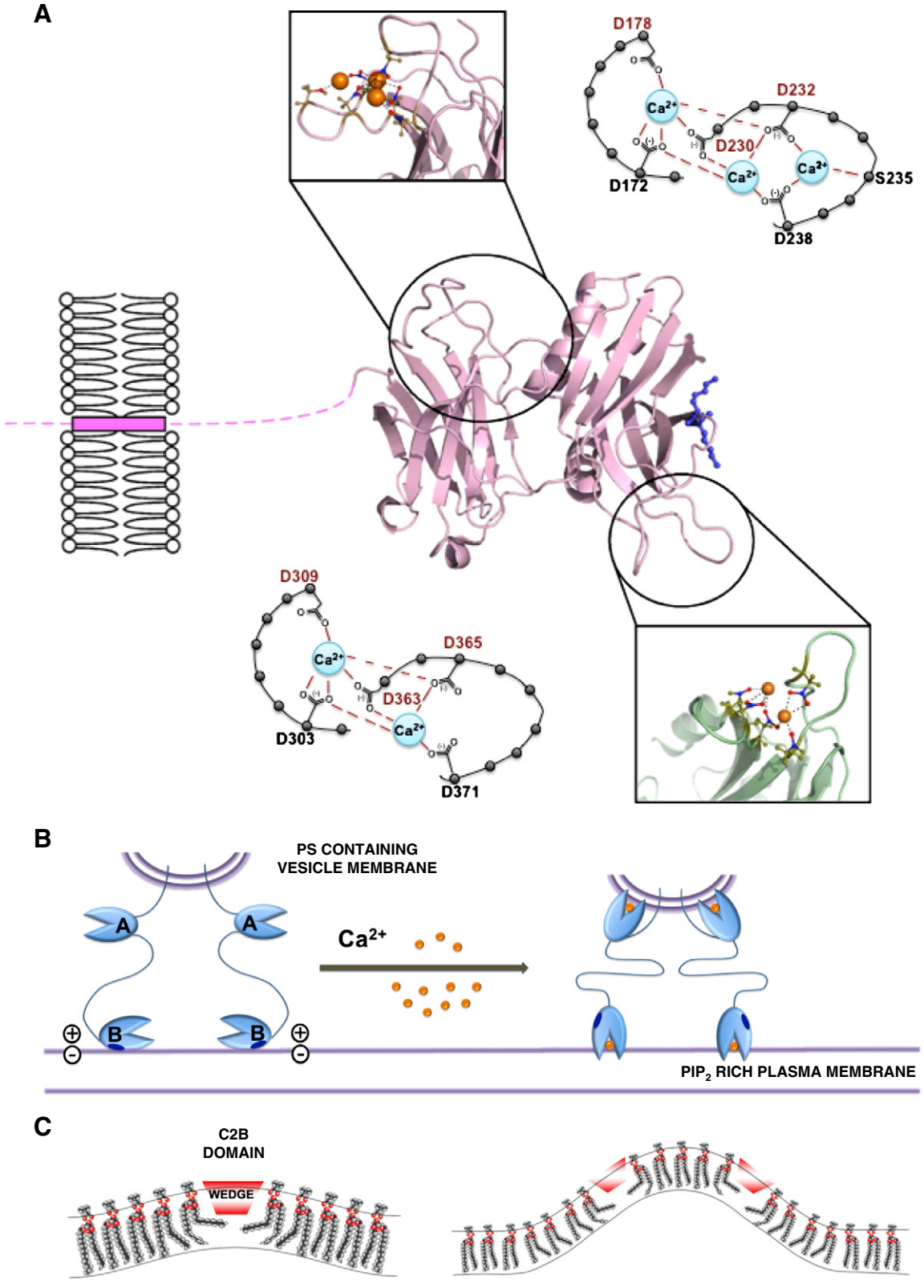
3.1. A specialized function of neurons: Ca^{2+}-dependent exocytosis

Exocytosis in neurons is unusual among membrane trafficking events, as it is strictly dependent on Ca^{2+}. The predominant Ca^{2+} sensor in neurons is thought to be synaptotagmin. The synaptotagmin family of proteins shares a common structure consisting of a N-terminal transmembrane region and two C-terminal C2 domains (termed C2A and C2B; see Fig. 3A and below). Synaptotagmins are highly conserved evolutionarily, and usually exist in multiple isoforms, even in model invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* [66]. In mammals, the synaptotagmin family now stands at 16 unique members, based on sequence homologies and function. Each isoform is encoded by a specific gene, while some (for example synaptotagmin 7) undergo additional alternative splicing [66]. Interestingly, the synaptotagmin isoforms all show differing Ca^{2+} affinities and expression patterns (including some synaptotagmins that localize to the plasma membrane), suggesting a substantial degree of adaptation in the nervous system. Three isoforms – synaptotagmins 1, 2 and 9 – have been unequivocally identified as localizing to synaptic vesicles and are proposed to act as Ca^{2+} sensors for synaptic vesicle exocytosis [67]. In this review, however, we limit ourselves to discussion of the original Ca^{2+} sensor synaptotagmin 1 (referred to as synaptotagmin for simplicity), for the simple reason that most work has been performed on this isoform.

Fig. 3. The role of PIP_2 and synaptotagmin in exocytosis. (A) Synaptotagmin is a synaptic vesicle protein. Synaptotagmin contains a N-terminal transmembrane region and two C-terminal C2 domains — C2A and C2B. The C2 domains are Ca^{2+} binding modules, which coordinate three and two Ca^{2+} ions, respectively. The acidic residues essential for binding Ca^{2+} are shown (orange spheres), with the residues mutated in known calcium binding mutants indicated (see insets). The polybasic region on the C2B domain is shown in blue. (B) A schematic model for the role of synaptotagmin in exocytosis. In the absence of Ca^{2+}, the C2A domain of synaptotagmin does not associate with membranes. The positioning of synaptic vesicles at putative fusion sites is likely due to the Ca^{2+}-independent interaction of the polybasic (lysine) patch on the C2 domain with PIP_2 (blue patch on C2B). Ca^{2+} influx into the terminal during an action potential leads to profound conformational changes in synaptotagmin; Ca^{2+} acts as a charge bridge minimizing electrostatic repulsion between the acidic Ca^{2+} binding residues on synaptotagmin and anionic membrane phospholipids, effectively allowing the hydrophobic amino acid residues that line the rims of the Ca^{2+}-binding pockets to penetrate lipid bilayers. The C2A domain preferentially penetrates regions of high PS content in synaptic vesicles, while the C2B domain penetrates regions enriched in PIP_2. Multiple synaptotagmin molecules are presumably involved in membrane penetration, perhaps facilitated by synaptotagmin clustering on the synaptic vesicle. (C) Penetration of membranes by multiple copies of synaptotagmin is thought to induce local membrane curvature — due to tilting of the local lipid acyl chains, essentially to compensate for the insertion of the C2 domain "wedge" and to maintain the hydrophobic core of the membrane. Such a region would be under high stress. It has been proposed that this stress is relieved during the lipid rearrangements that accompany the fusion process, reducing the overall energy cost of the reaction and favoring its completion.
Panel A adapted from [84]. Panel C based on [250].

3.1.1. Exocytosis: is synaptotagmin part of the core machinery?

Neurons share the core machinery for fusion with other cell types. It is now generally accepted that at the heart of every fusion process lie the so-called SNARE (Soluble NSF Attachment Receptor) proteins [3,68]. The three SNAREs classically viewed as involved in synaptic transmission are synaptobrevin 2/vesicle associated membrane protein 2 (VAMP2) on the synaptic vesicle, and syntaxin 1 and SNAP-25 on the plasma membrane. Extensive *in vitro* biochemical and biophysical analysis has allowed us to gain a detailed mechanistic understanding of this fusion machinery. SNAREs differ in both size and structure; for instance, synaptobrevin and syntaxin are both held at their respective membranes by single C-terminal transmembrane domains,



whereas SNAP-25 is attached to the membrane via four palmitoylated cysteines [69]. However, all SNAREs share one defining sequence — the SNARE motif. The SNARE motif is 60–70 amino acids in length, and includes eight heptad repeats that are indicative of coiled-coil motifs. Synaptobrevin and syntaxin both contain one SNARE motif, and SNAP-25 contains two. These SNARE motifs are largely unstructured in solution. However, when the SNAREs are brought together they assemble into a tight four-helical bundle — the so-called "core-complex". Assembly begins at the N-terminal end of the SNARE motifs and proceeds in a "zipper-like" fashion towards the C-terminal membrane anchors. It is thought that this "zippering" effectively bridges membranes, bringing them into close proximity and providing the energy needed for membrane fusion [3,68]. Major supporting evidence for the SNARE hypothesis comes from the fact that disruption of SNARE function, either by complete genetic ablation [70,71] or mutation of critical residues in the SNARE complex [72,73], severely disrupts Ca^{2+}-dependent secretion. In a similar manner, tetanus and botulinum toxins have been found to specifically cleave SNARE proteins and inhibit synaptic vesicle fusion [74–76].

One of the major questions remaining in neuroscience is how synaptotagmin senses the Ca^{2+} rise during an action potential and couples it to SNARE mediated fusion. Since the initial finding by Söllner and colleagues [77], many groups have reported an interaction between synaptotagmin and the SNARE complex. However, the mode of synaptotagmin action remains unclear. As SNAREs are thought to fuse membranes constitutively [78,79], a popular model has been that synaptotagmin acts on a partially preassembled SNARE complex, either directly as a "fusion clamp" [80,81], or by removing an accessory protein (for example complexin) that is functioning in this way. Such an explanation is attractive, given its intuitive simplicity. However, it also overlooks the fact that synaptotagmin contains two C2 domains.

3.1.2. C2 domains: the basis of synaptotagmin binding to PIP_2

C2 domains have been identified in a variety of membrane binding proteins, such as the conventional isoforms of protein kinase C (PKC), as Ca^{2+}/phospholipid binding modules. The C2 domains of synaptotagmin are no exception and can bind to acidic phospholipids in the presence of Ca^{2+} [82], particularly PIP_2 at the Ca^{2+} levels typically needed to trigger neurotransmitter release [83].

The basis of synaptotagmin's lipid binding can be found in the structure of the C2 domains — and both X-ray crystallography and NMR have been extensively applied to the problem.

Both C2 domains are structurally similar, consisting of ~130 amino acids, which adopt β-sandwich structures. The C2A domain binds three Ca^{2+} ions and the C2B domain binds two Ca^{2+} ions, respectively, through loops at the top of each β-sandwich [84,85]. Interestingly, the three-dimensional structures of C2 domains determined so far show no evidence that Ca^{2+} induces a substantial change from one well-defined conformation to another [86,87]. Rather it seems that Ca^{2+} causes a major change in the electrostatic potentials of the C2 domains, shielding negative charge and allowing the hydrophobic amino acids in the distal tips of the protein to penetrate lipid bilayers [88,89]. Interestingly, the C2 domains are thought to function as quasi-autonomous units; although Ca^{2+} binding to the individual C2 domains is thought to occur largely independently [84], membrane binding studies using recombinant synaptotagmin suggest that the C2B domain is heavily influenced in its membrane binding capabilities by the C2A domain (even when a Ca^{2+} binding mutant of C2A is used), indicating some co-operativity of action [57,84,90,91]. Likewise, correct membrane binding of the C2B domain is likely to affect C2A function by clamping synaptotagmin in the correct orientation [89] (and see below).

However, despite obvious structural similarities, several key differences are known to exist between the domains. These differences most likely explain why mutations in the C2B domain generally affect neurotransmitter release much more profoundly than those in C2A (see below). The most dramatic of these seems to be the difference in reported lipid preference, most likely reflecting subtle amino acid differences in the Ca^{2+}-binding loops [92]; C2A exhibits a preference for phosphatidylserine (PS), whereas C2B exhibits a preference for PIP_2 [57]. In addition, there are two lysine residues on the "side" of the C2B domain. These residues are thought to form a basic patch on C2B allowing pre-adsorption to PIP_2 prior to the Ca^{2+} signal, thus producing an enhanced coupling of synaptotagmin to the release machinery [57,59,93,94]. However, this site has also been proposed to mediate interactions with the SNARE proteins syntaxin and SNAP-25 in a Ca^{2+}-independent manner [95,96], and also to the full SNARE complex (although this interaction seems dependent on Ca^{2+}) [97–99]. It should also be noted that the C2A domain has been reported to bind to SNAP-25 within the SNARE complex in a Ca^{2+}-dependent manner, but with low affinity [100].

3.1.3. Membrane remodeling by C2 domains

An obvious question to ask is whether the binding of C2 domains to plasma membrane lipids can actually play a role in exocytosis; and if so, in what way do the C2 domains act? It has recently been proposed that synaptotagmin undergoes a large conformational change in the linker region between the C2 domains on binding to Ca^{2+} that may bring the vesicular and plasma membranes closer together. So does synaptotagmin function as merely a regulator of position and distance — binding PIP_2 at release sites, before bringing SNAREs close enough to initiate "zippering" in response to a Ca^{2+} signal [59,101]? Or can synaptotagmin perform "physical work" on membranes, as the Ca^{2+} binding loops of the C2 domains penetrate into the lipid core [88,89]? And, of equal importance, what is the relative balance between SNARE and lipid binding?

Until recently, it was impossible to assay the contribution of synaptotagmin lipid binding to exocytosis, as the vast majority of mutations that affect lipid binding also affect binding to SNAREs [102]. In addition, the multi-step (and vectorial) nature of synaptic vesicle transport makes deciphering the action of synaptotagmin particularly challenging; as both SNARE and lipid binding may actually play a role upstream of exocytosis, such as in vesicle docking to the plasma membrane, which would also result in an exocytic defect (as seen in synaptotagmin mutant neurons from *Drosophila* [103] and synaptotagmin null chromaffin cells [104]). Adding a further layer of complexity is the fact that synaptotagmin may have different functions depending on the biological system studied, as there do not appear to be any morphological defects in neurons cultured from synaptotagmin "knock-out" mice. However, this does need to be confirmed with additional quantitative ultrastructural studies [21].

To look specifically at the effects of lipid binding on the fusion process, the groups of McMahon and Chapman both took a similarly ingenious approach to the problem: they both used a reconstituted system with artificial vesicles (liposomes) containing the SNARE proteins and synaptotagmin to recapitulate membrane fusion [94,102]. Their basic rationale was that when synaptotagmin penetrates the membrane, the membrane should bend to accommodate the insertion and maintain its integrity. Essentially, this should produce a region of membrane instability that facilitates exocytosis. Small liposomes should be relatively unaffected by the induction of curvature stress, as they are highly curved already and prone to fusion. Large liposomes, however, are under much less curvature stress — so addition of synaptotagmin should induce tubulation and preferentially promote membrane fusion. Essentially both groups found synaptotagmin to facilitate fusion in such a manner, although the precise details of the process (such as whether both C2 domains are required and need to be tethered) remain disputed.

We have tried to formulate these results into a model of membrane remodeling during exocytosis, which is consistent with much of the previous work on synaptotagmin, to try and obtain a "consensus" view. Our model is summarized in Fig. 3B. In this model, before fusion the synaptic vesicle is held at the release site by a Ca^{2+}-independent

interaction of the C2B polybasic patch with PIP_2. As PIP_2 is known to cluster the SNARE protein syntaxin [34,35], it is possible that this might also lead to synaptotagmin–SNARE interactions, clamping the protein in a constrained conformation (not shown for simplicity) [105], which could also influence the position (and function) of the C2A domain [89]. On Ca^{2+}-influx, the C2B domain is in a position to reorientate in milliseconds and penetrate the PIP_2 containing membrane [57]. (It is thought the C2B domain penetrates up to a third of the way through the lipid monolayer [94]). As a result of this membrane insertion, the lipid polar heads will be displaced relative to the C2B domain, and to maintain membrane integrity the acyl chains will have to tilt. This tilting will then be propagated through neighboring lipids, inducing local bending of the monolayer in which the C2B domain is inserted. Due to monolayer coupling, the adjacent monolayer will follow, and the bilayer will bend as a functional unit. Although the insertion of a single C2 domain would only result in localized curvature, the effects of insertions are predicted to be additive. Two insertions close to each other, as shown, would result in high curvature between the inserted C2 domains (see Fig. 3C). Multiple insertions across a localized two-dimensional region would result in a local positive membrane curvature – membrane "buckling" – and the generation of a region under high stress. It has been proposed that this stress is relieved during the fusion process, reducing the overall energy cost of the reaction and favoring its completion [94,102].

At present, it is unclear to which membrane the C2A domain binds. Although in principal it could bind to either, we suggest that it is more likely to bind to the synaptic vesicle for two reasons. First, the crystal structure of the entire cytosolic domain of synaptotagmin in the absence of Ca^{2+} has revealed that the C2 domains face in opposite directions, hinting at the possibility that the molecule interacts with opposing membranes on Ca^{2+} influx [98]. Second, the vesicle membrane is known to contain high levels of PS [52], which would facilitate C2A binding [57,106]. Interestingly, however, insertion of the C2A domain into the membrane does not seem to induce curvature [102].

3.1.4. Is synaptotagmin membrane bending required in vivo?

The degree to which membrane curvature may aid the fusion process is a divisive question. Seminal experiments from the Rothman group established quite early that recombinant SNARE proteins alone, reconstituted into artificial membranes, could drive fusion *in vitro* [78]; a finding repeated by many labs [94,106–108], including when native, isolated synaptic vesicles were also used as one of the reaction partners [79,109]. In addition, work using SNARE cleaving toxins clearly indicates that SNAREs are needed for exocytosis *in vivo* [76].

So, is synaptotagmin induced membrane curvature *really* needed for exocytosis? Vesicle fusion is known to involve a large network of regulatory proteins at the active zone [110,111] and their effects on the bioenergetics of the fusion process are unknown. Thus, the contribution of membrane curvature may be an essential component to lowering the activation barrier for fusion sufficiently — and is consistent with the appearance of membrane invaginations in other membrane fusion events (for example, the exocytic release of inflammatory mediators from mast cell granules) [112]. In addition, it should be noted that neither the McMahon nor Chapman studies saw fusion in the absence of SNARE proteins, although fusion efficiency was greatly enhanced when synaptotagmin was present to induce membrane curvature.

In the future, the precise relationship of synaptotagmin membrane binding to SNARE function needs to be addressed. For instance, synaptotagmin may well act as a distance regulator, tethering membranes too far from each other for SNARE nucleation in the absence of Ca^{2+}, but bringing membranes close enough for membrane fusion in the presence of Ca^{2+} [59]. In this case a direct regulatory interaction with the SNARE proteins might not be needed for fusion. But the generation of a highly stressed membrane region during the process would no doubt facilitate fusion. Alternatively, local membrane distortion may act to facilitate fusion by causing conformational changes in the release machinery — either through direct synaptotagmin–SNARE interactions [113], or indirectly by changing protein orientations in the bilayer in an attempt to minimize hydrophobic mismatch [114]. All these possibilities remain to be fully explored.

Perhaps the most direct approach to assessing the role of membrane "buckling" during exocytosis is to "count" the number of synaptotagmins actually needed for fusion. Recent evidence using sophisticated imaging techniques to "count" SNARE complexes has suggested that a single SNARE complex may be enough to allow exocytosis, with no more than three being required for fast fusion in cultured hippocampal neurons or chromaffin cells [109,115,116]. It will be of great interest to use similar approaches to determine the number of synaptotagmins needed; only a single synaptotagmin would argue against our current models based on membrane curvature, which presumably require multiple proteins to generate and stabilize the required membrane curvature.

3.2. Endocytosis at the presynaptic terminal

3.2.1. The great endocytosis debate: "kiss-and-run" versus "clathrin-mediated endocytosis"

The general concept of vesicle recycling by endocytosis has been recognized since the early 1970s. Unfortunately, the precise details of the pathway(s) used are still the subject of controversy and fierce debate [117]. Two main mechanisms for endocytosis have been proposed — both with benefits and drawbacks for local vesicle recycling.

In the first pathway, "kiss-and-run", fusion with the plasma membrane never proceeds past the opening of a fusion pore, through which neurotransmitter is released. The vesicle is retrieved directly by closure of the fusion pore and recycled [118,119]. In a more extreme scenario, it has been proposed that the fusion pore is closed and the vesicle remains *in situ*, and is refilled with neurotransmitter at the active zone — so-called "kiss-and-stay" [120]. This model gained widespread attention given its intuitive attractiveness — allowing the recapture of the intact vesicle for subsequent rounds of exocytosis without further processing. However, direct retrieval would mean that old and/or faulty vesicle components would be constantly recycled in the presynaptic terminal.

In "clathrin-mediated endocytosis" it is generally thought that the synaptic vesicle collapses fully into the plasma membrane during exocytosis. Thus, it is necessary to selectively retrieve vesicular components from the complex mix of lipids and proteins in the plasma membrane. There is strong evidence that this occurs via the selection of vesicular components using specific adaptor proteins, which also act to recruit the protein clathrin to the membrane. Assembly of a clathrin cage ultimately acts to provide mechanical force to deform the membrane, with subsequent formation of a clathrin-coated vesicle [121–124]. After losing their clathrin coats, vesicles may recycle directly [125], or via an endosomal intermediate [126]. Although clathrin-mediated recycling seems on first reflection a less attractive mode of recycling than "kiss-and-run", the use of an endosomal recycling intermediate would likely act as a quality control mechanism, allowing the replacement of aged and/or damaged vesicle components (see Fig. 1A) [127].

Interestingly, these two modes of endocytosis need not be mutually exclusive and may both function in the same nerve terminal, depending on the strength and/duration of stimulus [128]. In this review, however, we shall limit ourselves to the role of clathrin-mediated endocytosis for two major reasons. First, capacitance measurements and fluorescence imaging have indicated that clathrin-mediated endocytosis plays *the major role* at many synapse types across species — from acutely isolated goldfish retinal bipolar cells [129] and cultured hippocampal neurons [124], through to the

Drosophila neuromuscular junction (NMJ) [130]. Second, both PIP_2 [131,132] and synaptotagmin [22,23,133] are thought to play crucial roles in this process at the synapse.

3.2.2. PIP_2 and AP2: a central hub in clathrin-mediated endocytosis

Extensive biochemical and cell biological analysis has revealed that clathrin-mediated endocytosis at the synapse depends on a complex web of protein–protein and protein–lipid interactions. In fact, the process shares many common components with the constitutive endocytic pathways that are responsible for internalizing transferrin, epidermal growth factor and low-density lipoprotein from the plasma membrane. (For a comprehensive overview of the interactions involved in clathrin-mediated endocytosis, the reader is directed to two recent reviews [4,134]).

Based on this data, it appears that the adaptor protein AP2 is at the heart of the network — acting as a central "*hub*" [4]. Its role essentially is two-fold. First, it is responsible for selecting vesicular components to be endocytosed. Second, it acts as a central scaffold for the recruitment of various accessory/regulatory factors involved in clathrin-coat formation [134].

The basis of AP2 function can be found in its structure. It consists of four subunits — α, β_2, μ_2 and σ_2. The 70 kDa trunk domains of α and β_2, together with the 50 kDa μ_2 and the 17 kDa σ_2 subunits, form the 200 kDa core of the protein that is responsible for membrane binding. The μ_2 and σ_2 subunits are also thought to be responsible for binding the cytoplasmic tails of cargo molecules in the plasma membrane (which are critical for defining the site of endocytosis on the membrane (see below)) [135,136]. The α and β_2 subunits both possess a 30 kDa bilobal C-terminal appendage; both of these appendages possess binding sites for various other proteins that need to be recruited from the cytoplasm to the membrane during endocytosis [132,137].

Given its central role in clathrin-mediated endocytosis and synaptic vesicle trafficking, activation of AP2 must be under tight temporal and spatial control. Recent X-ray crystallographic and biophysical data have demonstrated that AP2 can adopt two distinct conformations within the cell. In the cytoplasm, AP2 exists in a "locked" or "dead" conformation — in which the μ_2 and σ_2 subunits are sterically constrained by the β_2 subunit. *Recruitment to the plasma membrane is mediated by interactions of the α and β_2 subunits with PIP_2 in the plasma membrane.* Once membrane associated, a large conformational switch to an "unlocked" or "active" form occurs, due to a large-scale movement of the μ_2 subunit, which is also triggered by electrostatic attraction to PIP_2 in the plasma membrane (see Fig. 4A). This is consistent with live imaging studies in cultured BSC-1 cells in which PIP_2 was essential for defining and stabilizing endocytic sites [138]. Importantly, these endocytic sites may be further stabilized by a positive feedback loop established by AP2 binding to the membrane, which stimulates PIP_2 production via activation of PIPK1γ, and presumably promotes further recruitment and assembly of the endocytic apparatus [139]. The change in AP2 conformation that occurs on PIP_2 binding allows the σ_2 subunit to bind EDxxxLLI (dileucine) motifs (where x is any amino acid) in potential cargo proteins, while the μ_2 subunit binds via a β_2 augmentation to a tyrosine motif YxxΦ (where Φ is a hydrophobic amino acid) [132].

3.2.3. Coupling exo- and endocytosis: the role of synaptotagmin and stonin in endocytosis

Bioinformatics analysis of synaptic vesicle proteins has only identified dileucine-like and tyrosine-based endocytosis motifs in a limited number of proteins, such as the vesicular neurotransmitter transporters for glutamate, acetylcholine and monoamines [140,141], as well as SV2 [142]. This means that the majority of vesicle proteins apparently lack defined interactions with endocytic adaptor proteins (see Fig. 1B).

A case in point is the protein synaptotagmin — which is obviously essential for Ca^{2+}- triggered exocytic release. Although the C2B region of synaptotagmin is known to bind to the μ_2 subunit of AP2, this binding actually occurs through a region distinct from the traditional dileucine and tyrosine-based sorting signals [143]. However, it is likely to be the case that the binding of tyrosine-based motifs in SV2 facilitates synaptotagmin association with AP2 [142], reflected in the fact that gene "knock-out" of SV2 results in the accumulation of synaptotagmin on the plasma membrane, and reduced levels in synaptic vesicles [144]. In any case, binding to AP2 alone is not sufficient for synaptotagmin to be *efficiently* endocytosed. In contrast, to the synaptic vesicle proteins synaptophysin, synaptobrevin 2 and SV2 that are rapidly internalized when over-expressed in non-neuronal CHO fibroblasts [145], synaptotagmin remains surface localized [145,146]. Extensive mutational analysis has revealed that although synaptotagmin possesses an internalization signal in its C-terminal domain, this signal is "inhibited" by the C2B domain [146]. Obviously, this begs the question of what neuron specific signal interacts (either directly or indirectly) with the C2B domain to allow efficient endocytosis of synaptotagmin and correct reformation of synaptic vesicles?

Possible regulators of synaptotagmin internalization at synapses are stonins [26]. Originally identified in *Drosophila* as the protein stoned B, orthologs have since been found in the genomes of *C. elegans* (unc-41) and mammals (stonin 2); and all have been implicated in neurotransmission and/or SV recycling [15,147,148]. Here we focus on the role of mammalian stonin 2 (hereby referred to as stonin).

Extensive biochemical analysis involving co-immunoprecipitations and glutathione S-transferase (GST) pull-downs have established that stonin and synaptotagmin interact in lysates from brain and transfected cell lines [15,149,150]. Consistent with these findings, co-expression of synaptotagmin and stonin in both N1E-115 neuroblastoma cells and HEK293 cells was sufficient to recruit stonin to the plasma membrane through synaptotagmin binding [15]. Finally, in cultured hippocampal neurons, stonin facilitates the retrieval of a synaptotagmin reporter construct [15]. Importantly, stonin does not seem to facilitate endocytosis of dileucine and tyrosine motif containing proteins, such as the transferrin receptor, which undergo constitutive endocytosis [15].

Therefore, our current model of stonin action envisages it acting as a specific "bridge" between synaptotagmin and AP2, although the precise structural details of the complex remain unknown. Two principal points at which stonin interacts with the synaptotagmin-AP2 machinery have been identified. First, stonin interacts with basic determinants in the C2 domain of synaptotagmin (with C2A forming the major, but not exclusive, interacting surface) via a C-terminal μ-homology domain (μHD). This domain consists of approximately 350 amino acids and possesses roughly 30% sequence similarity to the μ_2 subunit of AP2 [151]. Importantly, however, it lacks the ability to both incorporate into *bona fide* AP2 complexes and to recognize tyrosine based cargo-binding motifs. Second, stonin binds to both the α-appendage and μ_2 subunits of AP2 via its N-terminal WxxF motifs [150]. Thus, the most intuitive explanation for the role of stonin is that it plays an essential role in strengthening the interaction between synaptotagmin and the endocytic machinery (see Fig. 4B). Whether this is the true function remains to be elucidated, as is the mechanism by which stonin overcomes the C2B "inhibitory" effect.

Consistent with this synaptotagmin-stonin-AP2 "bridging" hypothesis, over-expression of a WxxF mutant stonin in HEK cells reduced the internalization of co-expressed synaptotagmin by ~80% [15]. Stonin also has two high affinity NPF motifs (which for simplicity are not shown in Fig. 4B). These NPF motifs interact with Eps15 and intersectin [152]. Both these proteins are also thought to play a role in early clathrin-coat assembly, possibly by interaction with additional PIP_2 binding proteins: F-BAR domain-containing Fer/Cip4 homology domain-only proteins 1 and 2 (FCHo1/2). Interestingly, mutation of these NPF binding motifs, in addition to the WxxF mutation, *completely* abolished synaptotagmin internalization [15], consistent with the idea of a large self-stabilizing network of core proteins being required for endocytosis. In addition, targeting of the essential

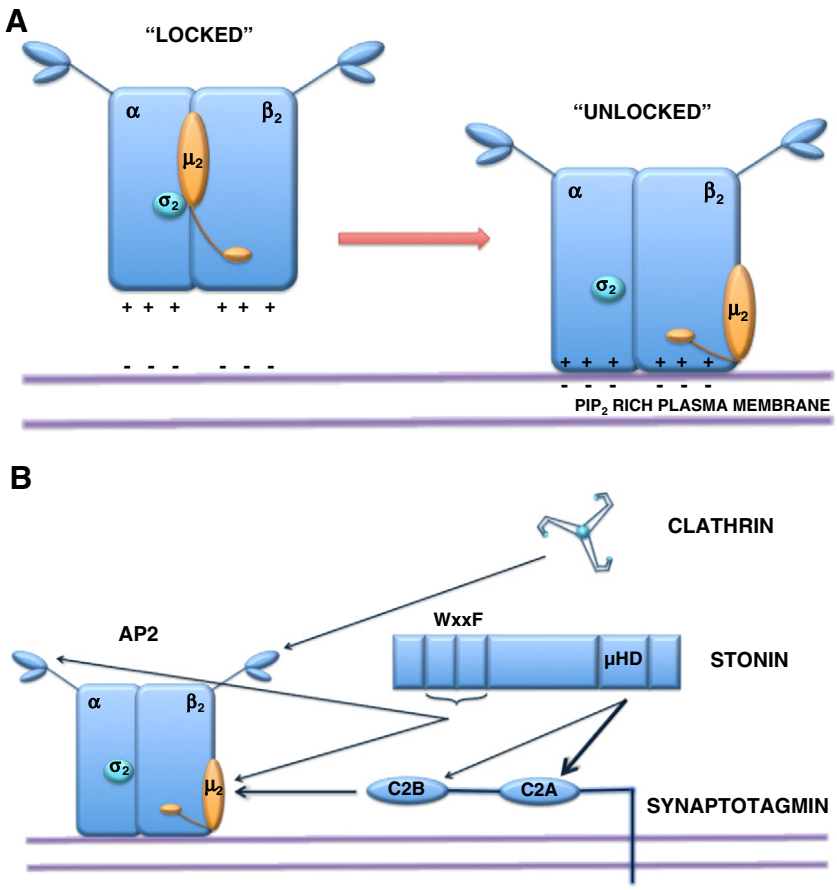


Fig. 4. The role of PIP_2 and the AP2 adaptor complex in endocytosis. (A) A schematic model for the membrane-induced conformational switching in AP2 that is necessary for cargo binding during clathrin-mediated endocytosis. In the cytosol, AP2 is present in an inactive "locked" conformation, which effectively prevents interactions with vesicle proteins. AP2 is initially recruited to the membrane by electrostatic interaction of its α and β_2 subunits with PIP_2. The switch to the active "unlocked" state is triggered by a large conformational change in the position of the μ_2 subunit, which is also driven by electrostatic attraction and binding to PIP_2 in the plasma membrane (not shown). (B) AP2 forms an essential membrane bound "hub" for clathrin-mediated endocytosis at the synapse. Synaptic vesicle proteins typically lack classical endocytic motifs. However, AP2 in its "unlocked" conformation can bind to the C2B domain of synaptotagmin via its μ_2 domain. Synaptotagmin is also the principal binding partner for the key neuronal protein stonin, which binds both C2 domains of synaptotagmin through an internal μ homology domain (μHD). Importantly, stonin association also serves to indirectly stabilize the interaction of synaptotagmin with AP2 — as stonin associates via its WxxF motifs with the α appendage and μ_2 subunit of AP2. Such an arrangement is attractive, given the proposed interactions of synaptotagmin with other core vesicle proteins, suggesting a basic mechanism for endocytosis in the absence of protein sorting motifs (see main text for details). The AP2-synaptotagmin-stonin interaction "hub" is one of the initial stages in a much larger set of protein–protein and protein–lipid interactions, which leads to clathrin-coat formation and the ultimate formation of clathrin-coated vesicles. The clathrin binding site on the β_2 appendage is illustrated as an example of this wider network of interactions; many other interactions have been omitted for clarity.
Panel A based on [132]. Panel B based on [26].

μ_2 subunit of AP2 using RNAi also resulted in both reduced internalization of exogenous synaptotagmin in HEK cells co-expressing stonin [15], and a significant decrease in the kinetics of endocytosis in cultured hippocampal neurons [153]. The lack of complete inhibition in these experiments is most likely related to the inefficiency of RNAi knockdown; even after more than 90% depletion by RNAi, AP2 can still be observed in a few clathrin cages forming on the membrane [154]. It seems that definitive analysis of the AP2-synaptotagmin-stonin "interactome" awaits genetic ablation studies.

Finally, stonin also possess a unique conserved domain of 140 amino acids termed the stonin homology domain (SHD). However, the function of this domain, and its relevance in endocytosis, remain unknown at present (also not shown in Fig. 4B).

4. Coupling exo- and endocytosis in the presynaptic terminal

4.1. Synaptotagmin: triggering a functional switch

The exact mechanism by which synaptotagmin converts from an exo- to an endocytic mode of action remains unknown; although it has to be considered likely that the C2 domains play a central role. Our lack of knowledge mostly reflects the technical difficulties inherent in isolating the two processes mechanistically. However, the newest generation of experimental tools (particularly the application of optical tracers to study neuronal activity) is starting to make an impact in the field [155].

The most thorough study to date makes use of cultured neurons from synaptotagmin null mice, which were transfected with various synaptotagmin constructs and their effects tested. In a clever piece of experimental design, these constructs were tagged with a pH sensitive variant of green fluorescent protein (pHluorin) at the luminal end of the protein [156]. Briefly, pHluorin marker systems make use of the fact that the vesicular lumen is acidic (see Fig. 1A). In the acidic pH of the vesicular lumen, pHluorins are quenched, and only become fluorescent when they are exposed to the more alkaline pH of the external culture media as a result of exocytosis. Hence, measuring the requenching rate as vesicles are reformed and subsequently reacidified is an effective measure of endocytosis [157]. A number of major points were raised by this study. First, Ca^{2+} was essential to the process — as mutated C2 domains that did not bind Ca^{2+} did not support endocytosis. Second, Ca^{2+} binding was not linked to membrane binding, as C2 domain mutations that showed abolished or enhanced membrane penetration had no effect on endocytosis (although a role for membrane association through the polybasic patch was not

ruled out). Third, both C2A and C2B in isolation were sufficient to support endocytosis — arguing that stonin association with one of the domains may be sufficiently strong to support endocytosis.

Interestingly, however, similar experiments performed in *Drosophila* indicated that the C2B domain plays the predominant role in endocytosis. In particular, the polybasic region is essential for regulating vesicle size, while the Ca^{2+} binding loops regulate the rate of endocytosis. The apparent discrepancy between these two studies might be due to the fact that the transgenic synaptotagmin constructs were inefficiently expressed and/or targeted to synaptic vesicles in *Drosophila* — which would have remained unclear as the pHluorin reporter used in this study was actually tagged to the luminal domain of synaptobrevin [158]. Or perhaps the different results reflect *bona fide* mechanistic differences within an evolutionarily conserved process (consistent with the different handling of specific vesicle proteins during recycling — see below) [140,159]. Irrespective, the finding that different regions of the C2 domains may differentially affect synaptic vesicle reformation indicates that a systematic analysis of both domains in various synaptic preparations is likely to provide a wealth of information regarding how synaptic vesicles are reformed.

Bearing these results in mind, here we present two possible models of how synaptotagmin might couple exo- and endocytosis (Fig. 5). In the first model, the insertion of synaptotagmin into the plasma membrane would cause a conformation change in synaptotagmin, possibly as a result of a hydrophobic mismatch in the transmembrane region, or the binding back of synaptotagmin onto the PIP_2 in the plasma membrane. Both mechanisms may result in a conformational change that allows AP2 and stonin binding (Fig. 5A). In the second model, synaptotagmin may need to interact with other endocytic partner proteins to form a functional complex. In this example, we use the known synaptotagmin binding partner SV2. SV2 is known to bind AP2 directly and to also promote the recruitment of synaptotagmin to AP2 (as discussed). Interestingly, the binding between these two proteins is inhibited by concentrations of Ca^{2+} in the low μM range — similar to those that are presumably found in the perisynaptic regions following an action potential [160]. Given that synaptotagmin binds and unbinds Ca^{2+} in milliseconds, it is still unclear whether Ca^{2+} binding to the C2 domains has any role in regulating this interaction [161]. Dissociation of the SV2-synaptotagmin complex might be needed to allow the binding of both proteins to AP2 and for the recruitment of stonin to stabilize the AP2-synaptotagmin complex (Fig. 5B). Of course, the models are not mutually exclusive and a combination of profound conformational changes and accessory partner binding is also possible. If synaptotagmin is itself part of a wider protein network (not shown), this would facilitate endocytosis in both models (see Section 4.2).

In addition, Ca^{2+} may also exert an effect by recruiting additional endocytic regulators to a complex. In addition to synaptotagmin, other candidate Ca^{2+} binding proteins at the synapse include calcineurin and calmodulin [5,162,163]. Phosphorylation of SV2 increases its interaction with synaptotagmin [164] — so Ca^{2+} dependent dephosphorylation (see above) would likewise play a role in increasing endocytosis. Differential expression and activation of various Ca^{2+}

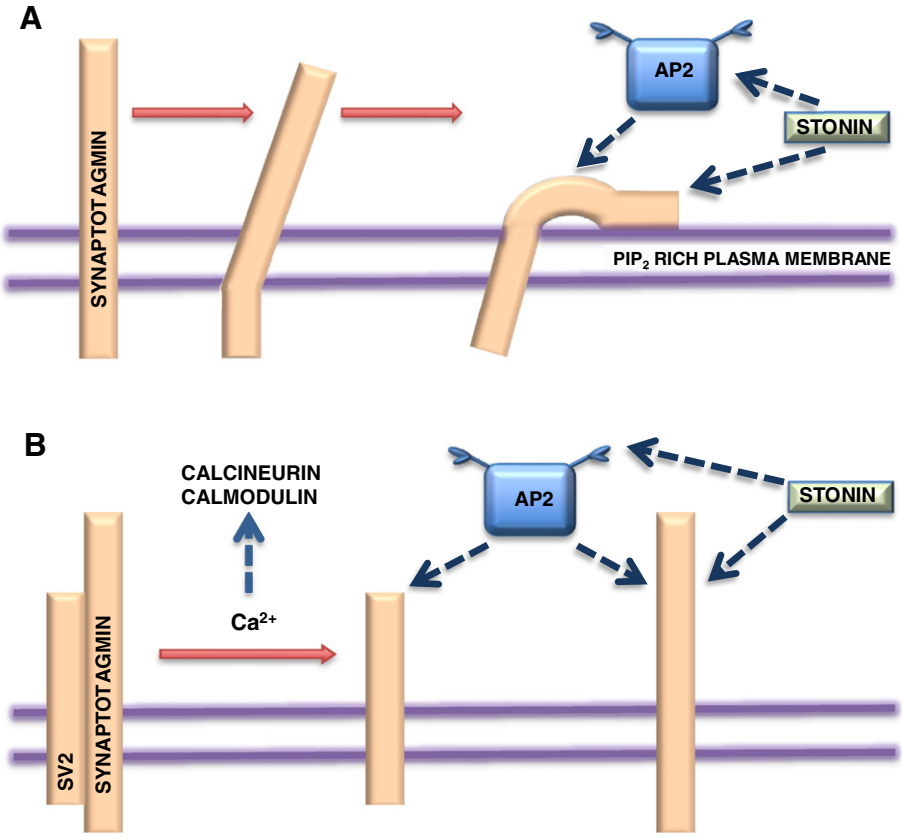


Fig. 5. Coupling exo- and endocytosis. A schematic model of how exo- and endocytosis might be coupled. Protein structure and size are not reflected in the scheme, which is intended to convey basic mechanisms by which the two processes could be linked. (A) Fusion of the synaptic vesicle with the plasma membrane alone may be sufficient to induce conformational changes in synaptotagmin necessary for endocytosis. For illustration purposes, synaptotagmin is initially shown as perpendicular to the membrane. Conformational changes may involve hydrophobic mismatch influencing the orientation of the transmembrane region, or binding of synaptotagmin back to PIP_2 on the plasma membrane via the polybasic patch. Such conformational changes may allow AP2 and stonin recruitment. (B) Synaptotagmin may need accessory proteins to engage the endocytic machinery. Synaptotagmin is known to bind SV2 in a reversible manner. This interaction is prevented in the presence of Ca^{2+}. SV2 is known to increase synaptotagmin binding to AP2 — so here we assume a 1:1:1 interaction between the proteins. Such a synaptotagmin-AP2 association could then be stabilized by stonin. The endocytic machinery might be the target of Ca^{2+} activated proteins such as calcineurin and calmodulin, which may also play a modulatory role in endocytosis. The models are not mutually exclusive — and endocytosis may require components from both models. The model is based mainly on information from [158] and [156]. In both models, synaptotagmin may be part of a wider network of associated proteins (see main text), which would be endocytosed in parallel.

binding proteins, in conjunction with the differential expression of their targets/binding partners, would also provide a satisfying explanation for the various effects of Ca^{2+} on endocytosis seen in different synaptic preparations. For instance, calcineurin has been proposed to promote endocytosis in cortical synaptosomes [5], but to inhibit endocytosis at the *Drosophila* NMJ [165].

4.2. Synaptotagmin: a co-ordinating scaffold for endocytosis?

Like synaptotagmin, the vast majority of vesicle proteins lack classical endocytic motifs. So the question arises of how these proteins are sorted during the recycling process. Although it remains possible that synaptic vesicle proteins are individually sorted during clathrin-mediated endocytosis (by as yet unidentified adaptors), this seems unlikely simply on the grounds of steric constraints. However, an attractive possibility, which has recently been proposed, is that synaptic vesicle components remain clustered at release sites (either by direct protein–protein interactions [166] or by cholesterol-dependent protein clustering [52,167]), and are then sorted as complete entities. Such a model has been proposed for synaptotagmin; biochemical studies, as well as super-resolution imaging studies using antibody staining of cultured neurons, have indicated that synaptotagmin may remain clustered after exocytosis [168,169], and thus be part of a wider network of interacting vesicle proteins [166] that facilitates endocytosis (although see below for references to contradictory findings). Such a model essentially makes synaptotagmin indispensible for maintaining the supply of "molecularly intact" vesicles that are available for re-release in the terminal — regardless of whether kinetic measurements of synaptotagmin mutants showed a complete block of endocytosis [23], or merely a slowing in the endocytic rate [22].

However, in our opinion, recovery of the whole vesicle via a network of synaptotagmin interactions is unlikely to be the sole mechanism used for three main reasons. First, analysis of stoned B in *Drosophila* indicates that the protein is involved in the selective retrieval of only a subset of synaptic vesicle proteins, including the glutamate transporter VGLUT1 [159]; in mammals, however, VGLUT1 retrieval is dependent on the protein endophilin [140]. Second, endocytosis was not completely blocked in synaptotagmin null mice. Third, retrieval of the SNARE protein synaptobrevin was recently shown to depend on interactions between the SNARE motif and the endocytic adaptors AP180 and clathrin assembly lymphoid myeloid leukemia protein (CALM) [170], presumably explaining the endocytic defects seen in the synaptobrevin "knock-out" mouse [171]. Of course, this suggests that multiple adaptors (and pathways) operate in parallel to accomplish faithful retrieval of a synaptic vesicle; although it still remains unclear whether such a mechanism would occur with complexes acting simultaneously, or whether they would act to produce distinct subsets of vesicles, such as those that contain amyloid precursor protein [172].

Additional proteins continue to be identified that play a role in endocytosis — although the precise molecular pathways involved, and their relative importance, remain to be elucidated. Such a case is the protein synaptophysin. Synaptophysin is a major component of mouse synaptic vesicles, which was recently shown to have a role in regulating endocytosis [173]. Yet the neurological phenotype of the synaptophysin "knock-out" mouse is actually extremely mild. In addition, synaptophysin is absent in *Drosophila* [174], arguing for a regulatory (rather than essential) role of synaptophysin in mammalian endocytosis.

The use of differential adaptor and recycling pathways is actually consistent with recent antibody labeling studies on isolated synaptic vesicles [175], which sought to determine the total number of protein copies per single vesicle (and hence derive a measure of variability). Although we think this study likely underestimates the number of proteins, due to steric constraints regarding the antibody labeling of intact vesicles [52], the results *are* consistent with the use of differential adaptor pathways to recycle protein components. On the one hand, a group of essential vesicle proteins including synaptotagmin, SV2, the proton pump ATPase and glutamate transporter VGLUT1 were found to be highly consistent from vesicle-to-vesicle indicating they recycle and are sorted with high precision. On the other, the exact copy numbers of proteins such as synaptobrevin and synaptophysin were found to vary considerably (see also Fig. 1B). At first sight, this seems particularly surprising in the case of synaptobrevin, which is an essential component of the fusion machinery. However, an average rat synaptic vesicle contains 70 copies of synaptobrevin [52], whereas in cultured hippocampal neurons only two copies seem necessary for vesicle fusion [116]. This suggests that in the absence of an efficient recycling pathway the system has compensated by increasing the copy numbers of essential vesicle proteins to ensure a comfortable "safety margin", effectively meaning that sufficient copies of each protein are "on board" each vesicle during repetitive rounds of recycling. This is also consistent with live-cell imaging studies using pHluorin-tagged synaptobrevin constructs, that showed a proportion of the protein to be both present and also highly mobile in the plasma membrane, indicative of diffusion into the plasma membrane following exocytosis [176–178]. However, these studies are controversial as pHluorin-tagged synaptotagmin was also found to reside in such a pool on the plasma membrane surface (contrast this behavior to the antibody-labeling study discussed previously, which suggested that synaptotagmin remains clustered at the plasma membrane [168]). A large effort has been made to solve this apparent contradiction; much debate has centered on whether the pHluorin-tagged synaptic vesicle proteins faithfully mimic their endogenous counterparts [169]. However, in our opinion, it is worth remembering that no biological pathway is ever going to operate with 100% precision — and particularly not when under conditions of high stress, such as intense neuronal stimulation. This inefficiency is well documented, and presumably explains why endosomal SNARE proteins and Rabs are found on a fraction of recycling synaptic vesicles [52,126]. Thus, if synaptic vesicles actually had to undergo postexocytic reclustering, this could be compromized under conditions of intense stimulation [169,179]. Further work, using a battery of synaptic proteins, in combination with various stimulation paradigms and recording techniques, will be necessary to fully understand this process.

Thus, while tremendous progress has obviously been made in understanding the general principals of endocytosis, major issues remain to be resolved, such as the role of adaptor complexes in tailoring distinct vesicle populations at certain types of synapses, under differing modes of stimulation [26,180]. Given the ubiquitous nature of AP2 function in endocytosis from the plasma membrane, and the plethora of known AP2 binding partners, we expect PIP_2 to continue to play a central role in this process.

5. After endocytosis: terminating the PIP_2 signal

The fate of lipids during exocytosis and endocytosis has still not been adequately addressed, presumably due to the technical challenges inherent in labeling and tracking single lipids [181,182]. Obviously, much more work needs to be done to address the spatio-temporal dynamics of this lipid at the active zone and how PIP_2 production correlates to its interaction with effectors (see Section 6). PIP_2 was undetectable in a study of isolated synaptic vesicles [52]. However, indirect evidence of PIP_2 action permits us to conclude that it must either partly exchange with the vesicle lipid during exocytosis, or at least maintain a "rim" around the collapsed vesicle, which is subsequently "collected" into the vesicle during endocytosis: as not only is clathrin cage assembly dependent on PIP_2, but also clathrin uncaging of reformed synaptic vesicles is thought to depend on PIP_2 hydrolysis by the inositol-5-phosphatase synaptojanin 1 [30]. In brain, synaptojanin 1 exists predominantly as a 145 kDa isoform (which is discussed further here) and a ubiquitously expressed 170 kDa isoform [183]). Synaptojanin is recruited to the membrane

at a late stage, after the clathrin coated pit has formed, via its interaction with the SH3 domain of the protein endophilin [184]. Endophilin itself interacts with the plasma membrane via its N-BAR domain [185], which has curvature-generating and -sensing properties [186,187], and presumably participates in the late-stage generation and maturation of the clathrin-coated pit [183]. Mechanistically, this makes a lot of sense as synaptojanin 1 mediated PIP_2 hydrolysis occurs preferentially at high curvature levels, such as those found on a synaptic vesicle [188], and would ultimately act as the catalyst for disassembly of the clathrin coat by disrupting the PIP_2–AP2 interaction (albeit in conjunction with the necessary co-factors Hsc70 and auxilin) [4,189]. Consistent with this interpretation, genetic ablation of the synaptojanin gene results in the accumulation of clathrin coated vesicles in the presynaptic terminal [30,189]. Interestingly, synaptojanin activity is also regulated by the activity of Cdk5. Cdk5 phosphorylates synaptojanin at serine 1144 (S1144), which inhibits its activity. Dephosphorylation by the Ca^{2+}-dependent phosphatase calcineurin results in enzyme activity and endophilin binding [190]. A tempting connection to make, therefore, is that the local Ca^{2+} signal generated during the action potential serves to co-ordinate the production and degradation of PIP_2 in the synaptic terminal. Of course, this does not preclude further actions of Ca^{2+} in the presynaptic terminal, which may also influence the efficiency of exo- and endocytosis (see Section 4.1).

6. Future perspectives

In this review, we concentrated on the role of PIP_2 at the synapse, because of its prominent role in exo- and endocytosis. However, PIP_2 is not the only neuroactive lipid. In fact, as our understanding of the nervous system increases, so does our appreciation that lipids are active players in mediating all aspects of synaptic activity. Perhaps, this is best illustrated by the increasing number of reviews on the subject of neuroactive lipids. Taken together, an overview of the complexity of lipid signaling in the nervous system can be found in several excellent reviews [191–195].

Unfortunately, studying lipids in living cells is currently a major challenge due to their very dynamic behavior. Fluorescent protein tags are not particularly suitable for such small molecules as lipids, as they often interfere with their localization and metabolism. To circumvent this issue, several methods to visualize lipids, either by producing fluorescently labeled lipid-binding proteins (see Section 2.1) or by direct labeling, have been developed (reviewed in [196]). Direct labeling approaches are particularly interesting, because they allow lipids to be studied in the native environment of the cell. One labeling technique of note is based on the principle of "click chemistry" — an approach for reliable chemical synthesis that typically involves an azide–alkyne cycloaddition reaction using copper as a catalyst [197–199]. These chemical groups do not occur naturally and for this reason click reactions are very specific. Cells are incubated with the alkyne-tagged lipid analog of interest, which resembles its natural counterpart and gets incorporated into the cell, and can then subsequently be labeled with a fluorophore bearing an azide moiety. This method allows the visualization of the lipid analog with high spatial and temporal resolution, while avoiding the mislocalization problems often caused by the fluorophore. Click chemistry has been successfully applied to visualize choline phospholipids in cultured cells, as well as on organs from mice injected with the choline analog [199]. However, one disadvantage of this method is that typically cells need to be fixed before labeling, due to the toxicity of the copper ions; and fixation prevents the study of dynamic processes. However, a few research groups have adapted this technique to be used on living cells [198,200].

Significant progress has also been made in developing techniques that can manipulate lipid levels in a regulated fashion in living cells. The use of photoactivatable protecting groups ("cages") allows manipulation of lipid levels in a temporally and spatially controlled fashion, by preventing biological activity of the lipid until the protecting groups are removed by a flash of light. Therefore, the "cages" effectively hinder rapid metabolism. Combined with cell-permeant groups, such as acetoxymethyl chemistry, these probes can enter cells and be activated [196]. A few caged lipids have already been synthesized [201] and the technique has been applied to manipulating phosphatidylinositol 3,4,5-trisphosphate levels in cultured cells [201].

Quick inactivation of a protein can be accomplished by adding a small molecule inhibitor that can easily cross the plasma membrane. Rapamycin [202], or its analog iRap [203], induces heterodimer formation between any two proteins, as long as these proteins have been modified to contain rapamycin-binding domains (protein domains from FK506 binding protein, FKBP, and from mTOR, FRB) and are present in the same compartment [204]. Rapamycin-induced heterodimerization has been successfully used to manipulate PIP_2 levels at the plasma membrane [205–207].

While the methods mentioned above have proved very useful in detecting, visualizing and manipulating lipids in cultured cells, research is limited by the need for *in vivo* models. This is particularly important as we are entering the post-genomic age [208–210], where we have the opportunity to identify the major lipid signaling pathways present at the synapse — but need adequate model systems in which to test our ideas. In fact, classical reverse genetics (with chemical mutagenesis and P-element transposition) has independently identified key roles for lipid signaling in the nervous systems of traditional model organisms, such as *C. elegans* and *D. melanogaster* [211,212]. Thus, it is highly likely that bioinformatics methods to mine genomic sequences together with genetic validation will rapidly increase the number of lipid signaling pathways identified in the nervous system [62,64,213]. In particular, comparative genomics across species seems especially suited to identify components of critical, conserved pathways [214]. A recent search of the fly genome for homologs of vertebrate proteins implicated in phosphoinositide metabolism not only found these proteins to be well conserved between mammals and flies, but also identified five additional proteins [215] (Slabbaert et al., Methods in Cell Biology, in press). Importantly, while mammalian genomes often encode several isoforms of a protein, the fruit fly genome harbors only one gene, greatly facilitating genetic studies.

As the majority of signaling pathways are very well conserved between flies and mammals, it is our opinion that *Drosophila* will be ideally suited for the future analysis of lipid function at the synapse *in vivo*. This is because *Drosophila* harbors an extended "genetic toolbox" that greatly simplifies genetic screening. In addition, the mushroom bodies of the *Drosophila* brain [216,217] and the "derivative synapse" of the neuromuscular junction (NMJ) [218,219] (see Fig. 6) are also accessible to the sophisticated analytical techniques needed to study neuronal function, such as electrophysiology, fluorescence imaging and electron microscopy. However, an important consideration in this respect must be that *Drosophila* cannot synthesize cholesterol *de novo*, similar to *C. elegans* [220].

Screening in *Drosophila* has traditionally involved interrupting correct gene expression over long timescales. Nowadays, this is most easily accomplished using one of several genome-wide RNAi libraries [221,222], in which hairpin constructs have been inserted transgenically into the genome under the control of the binary UAS/GAL4 system, allowing gene silencing in a tissue-specific manner [223]. Importantly, the most recent RNAi collections have been generated using specific docking sites, thereby reducing the potential for deleterious positional effects, as well as reducing the number of "off-targets". This system also enables a more reliable comparison between multiple RNAi lines. Temporal control can also be achieved by using a temperature sensitive mutant of the protein GAL80, which blocks GAL4 action at the low "permissive" temperature, but is inactive when shifted to higher temperatures, thereby allowing gene expression [224]. GAL80 is also used to repress GAL4 activity in the Mosaic Analysis with a Repressible Cell Marker (MARCM) system —

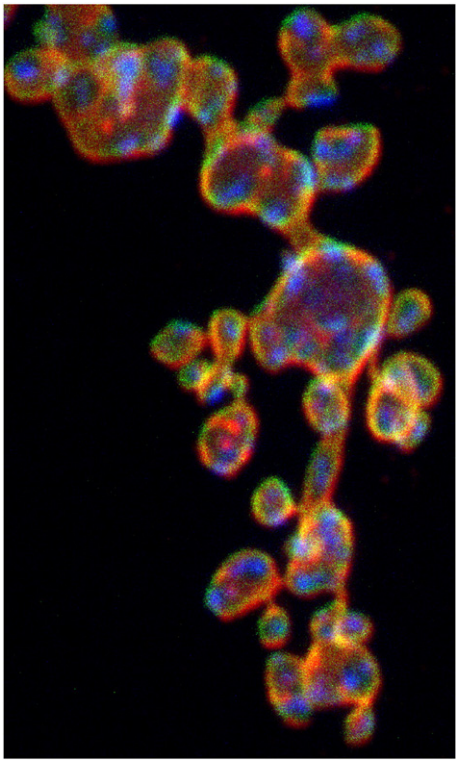


Fig. 6. Genetic studies of lipid signaling. Image of a neuromuscular junction from a third instar *Drosophila* larvae. In this example, the lipid binding pleckstrin homology (PH) domain of phospholipase C was fused to green fluorescent protein (PLC-GFP) and integrated into the genome as a UAS-transgene. Expression has been driven constitutively in neurons using the pan-neuronal nSyb-Gal4 driver to label regions high in PIP_2 (green). Neurons are labeled with an antibody against the neuron specific membrane protein HRP (red), and active zones marked with an antibody against the *Drosophila* active zone protein Bruchpilot (blue).

a valuable method for labeling and manipulating single cells [225]. Other binary systems, such as the lexA plot and the Q system, have also been developed, allowing parallel genetic manipulation of different cell populations [226,227].

However, although RNAi mediated knock down is fast and simple to perform, incomplete gene inactivation is a major concern (Slabbaert, Methods in Cell Biology, in press). Therefore "classical mutations" are also still widely used (see above). These include transposable genetic elements [228,229], and chemical mutagens such as ethyl methanesulfonate (EMS) [230] — although these have the drawback of disrupting gene expression in all tissues. In addition, homologous recombination [231] and recombination mediated cassette exchange [232,233] are also viable alternatives for gene disruption.

Lipids play multiple roles in a variety of signaling pathways, and prolonged activation or inactivation of lipid modifying/interacting proteins using genetic disruption may mask the acute effects of a particular lipid on cellular physiology — particularly if localized activation is required. To circumvent these problems, technologies also now exist to disrupt protein function acutely *in vivo*. Principal among these is the FlAsH-FALI technique [234–236]. This is based on the use of a tetracysteine (4 C) sequence engineered into the protein of interest. This tetracysteine sequence binds with high affinity to the membrane permeable fluorescein derivative FlAsH-EDT_2. Excitation of FlAsH with ~500 nm light results in the production of reactive oxygen species within a radius of a few angstroms, which inactivate the tagged protein within seconds. Again, use of the UAS/GAL4 transgenic system allows tissue-specific expression of the tetracysteine protein. In *Drosophila*, the use of FlAsH technology has proved particularly useful in investigating exo- and endocytic coupling. In *Drosophila*, reversible inhibition of endocytosis can be achieved by using mutations in the fly homologue of the GTPase dynamin, shibire, which prevent membrane scission during endocytosis; eventually leading to a depletion of synaptic vesicles at the synapse and paralysis [237]. Endocytosis can be blocked either in the whole organism by using the temperature sensitive shibire mutant, or in a population of cells by overexpressing a dominant negative form of the shibire protein under GAL4/UAS control. Hence, shibire effectively allow exocytosis to be uncoupled from endocytosis. In particular, the temperature sensitive form of shibire has proved particularly powerful when used in combination with FlAsH-FALI. Shibire mutant flies are moved to the restrictive temperature where endocytosis is blocked and the protein of interest inactivated by FlAsH-FALI, before the flies are returned to the permissive temperature and the effects on endocytosis assayed. In this way, a role for synaptotagmin in endocytosis was identified in *Drosophila* (independent of its role in exocytosis) [23]. Potentially, this combination of methods could be extended to any protein of interest, although it should be noted at this point that the production of free radicals during FlAsH-FALI may result in non-specific damage. Quite possibly this may explain why there is a complete block of endocytosis after synaptotagmin inactivation in *Drosophila*, whereas genetic ablation in mouse only leads to a slowing of endocytosis [22].

In combination, these methods (alongside the continuous introduction of new genetic technologies) should present a powerful toolbox to elucidate the *in vivo* function of lipids at the synapse. Importantly, a comprehensive list of *Drosophila* lines available for defined genetic loci is held at Flybase (http://flybase.org). And the vast majority of these are lines available from public stock centers, such as the Vienna Drosophila RNAi Center (http://stockcenter.vdrc.at) and Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu/), which should greatly accelerate future lipid research.

7. Summary

In this review, we have attempted to illustrate the pivotal role lipids play in the nervous system by considering the function of PIP_2 in the presynaptic terminal. During vesicle cycling, we believe PIP_2 plays a major (if not the key) role in coupling exo-and endocytosis, ensuring a constant supply of usable vesicles for synaptic transmission. So far, analysis has been mainly limited to *in vitro* biochemical and cell-biological techniques. But with the advent of powerful genetic techniques, the roles of PIP_2 and its associated binding proteins are starting to be investigated *in vivo*. As the number of lipid signaling pathways identified in neurons increases, we expect application of these approaches to be equally valid and to revolutionize our understanding of synapse function.

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References

[1] T.C. Südhof, The synaptic vesicle cycle revisited, Neuron 28 (2000) 317–320.
[2] T.C. Südhof, The synaptic vesicle cycle: a cascade of protein–protein interactions, Nature 375 (1995) 645–653.
[3] R. Jahn, R.H. Scheller, SNAREs — engines for membrane fusion, Nat. Rev. Mol. Cell Biol. 7 (2006) 631–643.

[4] H.T. McMahon, E. Boucrot, Molecular mechanism and physiological functions of clathrin-mediated endocytosis, Nat. Rev. Mol. Cell Biol. 12 (2011) 517–533.
[5] B. Marks, H.T. McMahon, Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals, Curr. Biol. 8 (1998) 740–749.
[6] X.S. Wu, B.D. McNeil, J. Xu, J. Fan, L. Xue, E. Melicoff, R. Adachi, L. Bai, L.G. Wu, Ca^{2+} and calmodulin initiate all forms of endocytosis during depolarization at a nerve terminal, Nat. Neurosci. 12 (2009) 1003–1010.
[7] N. Hosoi, M. Holt, T. Sakaba, Calcium dependence of exo- and endocytotic coupling at a glutamatergic synapse, Neuron 63 (2009) 216–229.
[8] M.A. Cousin, P.J. Robinson, The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis, Trends Neurosci. 24 (2001) 659–665.
[9] H. von Gersdorff, G. Matthews, Inhibition of endocytosis by elevated internal calcium in a synaptic terminal, Nature 370 (1994) 652–655.
[10] M.A. Cousin, P.J. Robinson, Ca^{2+} influx inhibits dynamin and arrests synaptic vesicle endocytosis at the active zone, J. Neurosci. 20 (2000) 949–957.
[11] J. Leitz, E.T. Kavalali, Ca^{2+} influx slows single synaptic vesicle endocytosis, J. Neurosci. 31 (2011) 16318–16326.
[12] F. Kawasaki, M. Hazen, R.W. Ordway, Fast synaptic fatigue in shibire mutants reveals a rapid requirement for dynamin in synaptic vesicle membrane trafficking, Nat. Neurosci. 3 (2000) 859–860.
[13] J.Y. Sun, X.S. Wu, L.G. Wu, Single and multiple vesicle fusion induce different rates of endocytosis at a central synapse, Nature 417 (2002) 555–559.
[14] E.L. Clayton, G.J. Evans, M.A. Cousin, Bulk synaptic vesicle endocytosis is rapidly triggered during strong stimulation, J. Neurosci. 28 (2008) 6627–6632.
[15] M.K. Diril, M. Wienisch, N. Jung, J. Klingauf, V. Haucke, Stonin 2 is an AP2-dependent endocytic sorting adaptor for synaptotagmin internalization and recycling, Dev. Cell 10 (2006) 233–244.
[16] H. von Gersdorff, G. Matthews, Dynamics of synaptic vesicle fusion and membrane retrieval in synaptic terminals, Nature 367 (1994) 735–739.
[17] G. Neves, L. Lagnado, The kinetics of exocytosis and endocytosis in the synaptic terminal of goldfish retinal bipolar cells, J. Physiol. 515 (Pt 1) (1999) 181–202.
[18] E. Macia, M. Ehrlich, R. Massol, E. Boucrot, C. Brunner, T. Kirchhausen, Dynasore, a cell-permeable inhibitor of dynamin, Dev. Cell 10 (2006) 839–850.
[19] I.G. Mills, G.J. Praefcke, Y. Vallis, B.J. Peter, L.E. Olesen, J.L. Gallop, P.J. Butler, P.R. Evans, H.T. McMahon, EpsinR: an AP1/clathrin interacting protein involved in vesicle trafficking, J. Cell Biol. 160 (2003) 213–222.
[20] S.P. Davies, H. Reddy, M. Caivano, P. Cohen, Specificity and mechanism of action of some commonly used protein kinase inhibitors, Biochem. J. 351 (2000) 95–105.
[21] E.R. Chapman, How does synaptotagmin trigger neurotransmitter release? Annu. Rev. Biochem. 77 (2008) 615–641.
[22] K. Nicholson-Tomishima, T.A. Ryan, Kinetic efficiency of endocytosis at mammalian CNS synapses requires synaptotagmin I, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 16648–16652.
[23] K.E. Poskanzer, K.W. Marek, S.T. Sweeney, G.W. Davis, Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis *in vivo*, Nature 426 (2003) 559–563.
[24] S. McLaughlin, J. Wang, A. Gambhir, D. Murray, PIP_2 and proteins: interactions, organization, and information flow, Annu. Rev. Biophys. Biomol. Struct. 31 (2002) 151–175.
[25] G. Di Paolo, P. De Camilli, Phosphoinositides in cell regulation and membrane dynamics, Nature 443 (2006) 651–657.
[26] T. Maritzen, J. Podufall, V. Haucke, Stonins — specialized adaptors for synaptic vesicle recycling and beyond? Traffic 11 (2010) 8–15.
[27] M.R. Wenk, L. Lucast, G. Di Paolo, A.J. Romanelli, S.F. Suchy, R.L. Nussbaum, G.W. Cline, G.I. Shulman, W. McMurray, P. De Camilli, Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry, Nat. Biotechnol. 21 (2003) 813–817.
[28] P.J. Wen, S.L. Osborne, F.A. Meunier, Dynamic control of neuroexocytosis by phosphoinositides in health and disease, Prog. Lipid Res. 50 (2011) 52–61.
[29] M.A. De Matteis, A. Godi, PI-loting membrane traffic, Nat. Cell Biol. 6 (2004) 487–492.
[30] O. Cremona, G. Di Paolo, M.R. Wenk, A. Lüthi, W.T. Kim, K. Takei, L. Daniell, Y. Nemoto, S.B. Shears, R.A. Flavell, D.A. McCormick, P. De Camilli, Essential role of phosphoinositide metabolism in synaptic vesicle recycling, Cell 99 (1999) 179–188.
[31] K.D. Micheva, R.W. Holz, S.J. Smith, Regulation of presynaptic phosphatidylinositol 4,5-biphosphate by neuronal activity, J. Cell Biol. 154 (2001) 355–368.
[32] K. Simons, M.J. Gerl, Revitalizing membrane rafts: new tools and insights, Nat. Rev. Mol. Cell Biol. 11 (2010) 688–699.
[33] I. Milosevic, J.B. Sørensen, T. Lang, M. Krauss, G. Nagy, V. Haucke, R. Jahn, E. Neher, Plasmalemmal phosphatidylinositol-4,5-bisphosphate level regulates the releasable vesicle pool size in chromaffin cells, J. Neurosci. 25 (2005) 2557–2565.
[34] D.J. James, C. Khodthong, J.A. Kowalchyk, T.F. Martin, Phosphatidylinositol 4,5-bisphosphate regulates SNARE-dependent membrane fusion, J. Cell Biol. 182 (2008) 355–366.
[35] G. van den Bogaart, K. Meyenberg, H.J. Risselada, H. Amin, K.I. Willig, B.E. Hubrich, M. Dier, S.W. Hell, H. Grubmüller, U. Diederichsen, R. Jahn, Membrane protein sequestering by ionic protein–lipid interactions, Nature 479 (2011) 552–555.
[36] Y. Hua, R. Sinha, C.S. Thiel, R. Schmidt, J. Hüve, H. Martens, S.W. Hell, A. Egner, J. Klingauf, A readily retrievable pool of synaptic vesicles, Nat. Neurosci. 14 (2011) 833–839.
[37] R.L. Doughman, A.J. Firestone, R.A. Anderson, Phosphatidylinositol phosphate kinases put $PI(4,5)P_2$ in its place, J. Membr. Biol. 194 (2003) 77–89.
[38] M.R. Wenk, L. Pellegrini, V.A. Klenchin, G. Di Paolo, S. Chang, L. Daniell, M. Arioka, T.F. Martin, P. De Camilli, PIP kinase Igamma is the major $PI(4,5)P_2$ synthesizing enzyme at the synapse, Neuron 32 (2001) 79–88.
[39] N. Kahlfeldt, A. Vahedi-Faridi, S.J. Koo, J.G. Schäfer, G. Krainer, S. Keller, W. Saenger, M. Krauss, V. Haucke, Molecular basis for association of PIPKI gamma-p90 with clathrin adaptor AP-2, J. Biol. Chem. 285 (2010) 2734–2749.
[40] L.A. Volpicelli-Daley, L. Lucast, L.W. Gong, L. Liu, J. Sasaki, T. Sasaki, C.S. Abrams, Y. Kanaho, P. De Camilli, Phosphatidylinositol-4-phosphate 5-kinases and phosphatidylinositol 4,5-bisphosphate synthesis in the brain, J. Biol. Chem. 285 (2010) 28708–28714.
[41] G. Di Paolo, H.S. Moskowitz, K. Gipson, M.R. Wenk, S. Voronov, M. Obayashi, R. Flavell, R.M. Fitzsimonds, T.A. Ryan, P. De Camilli, Impaired $PtdIns(4,5)P_2$ synthesis in nerve terminals produces defects in synaptic vesicle trafficking, Nature 431 (2004) 415–422.
[42] J.R. Morgan, G. Di Paolo, H. Werner, V.A. Shchedrina, M. Pypaert, V.A. Pieribone, P. De Camilli, A role for talin in presynaptic function, J. Cell Biol. 167 (2004) 43–50.
[43] M.R. Wenk, P. De Camilli, Protein–lipid interactions and phosphoinositide metabolism in membrane traffic: insights from vesicle recycling in nerve terminals, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 8262–8269.
[44] A. Honda, M. Nogami, T. Yokozeki, M. Yamazaki, H. Nakamura, H. Watanabe, K. Kawamoto, K. Nakayama, A.J. Morris, M.A. Frohman, Y. Kanaho, Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation, Cell 99 (1999) 521–532.
[45] Y. Funakoshi, H. Hasegawa, Y. Kanaho, Regulation of PIP5K activity by Arf6 and its physiological significance, J. Cell. Physiol. 226 (2011) 888–895.
[46] M. Krauss, M. Kinuta, M.R. Wenk, P. De Camilli, K. Takei, V. Haucke, ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type Igamma, J. Cell Biol. 162 (2003) 113–124.
[47] A. Béglé, P. Tryoen-Tóth, J. de Barry, M.F. Bader, N. Vitale, ARF6 regulates the synthesis of fusogenic lipids for calcium-regulated exocytosis in neuroendocrine cells, J. Biol. Chem. 284 (2009) 4836–4845.
[48] A. Moritz, P.N. De Graan, W.H. Gispen, K.W. Wirtz, Phosphatidic acid is a specific activator of phosphatidylinositol-4-phosphate kinase, J. Biol. Chem. 267 (1992) 7207–7210.
[49] G.H. Jenkins, P.L. Fisette, R.A. Anderson, Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid, J. Biol. Chem. 269 (1994) 11547–11554.
[50] Y. Humeau, N. Vitale, S. Chasserot-Golaz, J.L. Dupont, G. Du, M.A. Frohman, M.F. Bader, B. Poulain, A role for phospholipase D1 in neurotransmitter release, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 15300–15305.
[51] J. Guo, M.R. Wenk, L. Pellegrini, F. Onofri, F. Benfenati, P. De Camilli, Phosphatidylinositol 4-kinase type IIα is responsible for the phosphatidylinositol 4-kinase activity associated with synaptic vesicles, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 3995–4000.
[52] S. Takamori, M. Holt, K. Stenius, E.A. Lemke, M. Grønborg, D. Riedel, H. Urlaub, S. Schenck, B. Brügger, P. Ringler, S.A. Müller, B. Rammner, F. Gräter, J.S. Hub, B.L. De Groot, G. Mieskes, Y. Moriyama, J. Klingauf, H. Grubmüller, J. Heuser, F. Wieland, R. Jahn, Molecular anatomy of a trafficking organelle, Cell 127 (2006) 831–846.
[53] N. Brose, C. Rosenmund, Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters, J. Cell Sci. 115 (2002) 4399–4411.
[54] P. Viard, A.J. Butcher, G. Halet, A. Davies, B. Nürnberg, F. Heblich, A.C. Dolphin, PI3K promotes voltage-dependent calcium channel trafficking to the plasma membrane, Nat. Neurosci. 7 (2004) 939–946.
[55] B.N. Kholodenko, J.B. Hoek, H.V. Westerhoff, Why cytoplasmic signalling proteins should be recruited to cell membranes, Trends Cell Biol. 10 (2000) 173–178.
[56] M.A. McCloskey, M.M. Poo, Rates of membrane-associated reactions: reduction of dimensionality revisited, J. Cell Biol. 102 (1986) 88–96.
[57] J. Bai, W.C. Tucker, E.R. Chapman, PIP_2 increases the speed of response of synaptotagmin and steers its membrane-penetration activity toward the plasma membrane, Nat. Struct. Mol. Biol. 11 (2004) 36–44.
[58] S. McLaughlin, D. Murray, Plasma membrane phosphoinositide organization by protein electrostatics, Nature 438 (2005) 605–611.
[59] G. van den Bogaart, S. Thutupalli, J.H. Risselada, K. Meyenberg, M. Holt, D. Riedel, U. Diederichsen, S. Herminghaus, H. Grubmüller, R. Jahn, Synaptotagmin-1 may be a distance regulator acting upstream of SNARE nucleation, Nat. Struct. Mol. Biol. 18 (2011) 805–812.
[60] D. Araç, X. Chen, H.A. Khant, J. Ubach, S.J. Ludtke, M. Kikkawa, A.E. Johnson, W. Chiu, T.C. Südhof, J. Rizo, Close membrane-membrane proximity induced by Ca^{2+}-dependent multivalent binding of synaptotagmin-1 to phospholipids, Nat. Struct. Mol. Biol. 13 (2006) 209–217.
[61] G. Rohde, D. Wenzel, V. Haucke, A phosphatidylinositol (4,5)-bisphosphate binding site within mu2-adaptin regulates clathrin-mediated endocytosis, J. Cell Biol. 158 (2002) 209–214.
[62] M.A. Lemmon, Membrane recognition by phospholipid-binding domains, Nat. Rev. Mol. Cell Biol. 9 (2008) 99–111.
[63] K. Salim, M.J. Bottomley, E. Querfurth, M.J. Zvelebil, I. Gout, R. Scaife, R.L. Margolis, R. Gigg, C.I. Smith, P.C. Driscoll, M.D. Waterfield, G. Panayotou, Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase, EMBO J. 15 (1996) 6241–6250.
[64] J.P. DiNitto, D.G. Lambright, Membrane and juxtamembrane targeting by PH and PTB domains, Biochim. Biophys. Acta 1761 (2006) 850–867.
[65] T.W. Harris, E. Hartwieg, H.R. Horvitz, E.M. Jorgensen, Mutations in synaptojanin disrupt synaptic vesicle recycling, J. Cell Biol. 150 (2000) 589–600.
[66] Z.P. Pang, T.C. Südhof, Cell biology of Ca^{2+}-triggered exocytosis, Curr. Opin. Cell Biol. 22 (2010) 496–505.

[67] J. Xu, T. Mashimo, T.C. Südhof, Synaptotagmin-1, -2, and -9: Ca^{2+} sensors for fast release that specify distinct presynaptic properties in subsets of neurons, Neuron 54 (2007) 567–581.
[68] R. Jahn, T. Lang, T.C. Südhof, Membrane fusion, Cell 112 (2003) 519–533.
[69] G.R. Prescott, O.A. Gorleku, J. Greaves, L.H. Chamberlain, Palmitoylation of the synaptic vesicle fusion machinery, J. Neurochem. 110 (2009) 1135–1149.
[70] S. Schoch, F. Deák, A. Königstorfer, M. Mozhayeva, Y. Sara, T.C. Südhof, E.T. Kavalali, SNARE function analyzed in synaptobrevin/VAMP knockout mice, Science 294 (2001) 1117–1122.
[71] P. Washbourne, P.M. Thompson, M. Carta, E.T. Costa, J.R. Mathews, G. Lopez-Benditó, Z. Molnár, M.W. Becher, C.F. Valenzuela, L.D. Partridge, M.C. Wilson, Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis, Nat. Neurosci. 5 (2002) 19–26.
[72] J.P. Weber, K. Reim, J.B. Sørensen, Opposing functions of two sub-domains of the SNARE-complex in neurotransmission, EMBO J. 29 (2010) 2477–2490.
[73] K. Wiederhold, T.H. Kloepper, A.M. Walter, A. Stein, N. Kienle, J.B. Sørensen, D. Fasshauer, A coiled coil trigger site is essential for rapid binding of synaptobrevin to the SNARE acceptor complex, J. Biol. Chem. 285 (2010) 21549–21559.
[74] G. Schiavo, F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B.R. DasGupta, C. Montecucco, Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin, Nature 359 (1992) 832–835.
[75] J. Blasi, E.R. Chapman, S. Yamasaki, T. Binz, H. Niemann, R. Jahn, Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin, EMBO J. 12 (1993) 4821–4828.
[76] G. Schiavo, M. Matteoli, C. Montecucco, Neurotoxins affecting neuroexocytosis, Physiol. Rev. 80 (2000) 717–766.
[77] T. Söllner, M.K. Bennett, S.W. Whiteheart, R.H. Scheller, J.E. Rothman, A protein assembly-disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion, Cell 75 (1993) 409–418.
[78] T. Weber, B.V. Zemelman, J.A. McNew, B. Westermann, M. Gmachl, F. Parlati, T.H. Söllner, J.E. Rothman, SNAREpins: minimal machinery for membrane fusion, Cell 92 (1998) 759–772.
[79] M. Holt, D. Riedel, A. Stein, C. Schuette, R. Jahn, Synaptic vesicles are constitutively active fusion machines that function independently of Ca^{2+}, Curr. Biol. 18 (2008) 715–722.
[80] K.C. Martin, Y. Hu, B.A. Armitage, S.A. Siegelbaum, E.R. Kandel, B.K. Kaang, Evidence for synaptotagmin as an inhibitory clamp on synaptic vesicle release in Aplysia neurons, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 11307–11311.
[81] N. Sasakawa, M. Ohara-Imaizumi, M. Fukuda, H. Kabayama, K. Mikoshiba, K. Kumakura, Dissociation of inositol polyphosphates from the C2B domain of synaptotagmin facilitates spontaneous release of catecholamines in adrenal chromaffin cells. A suggestive evidence of a fusion clamp by synaptotagmin, Neuropharmacology 60 (2011) 1364–1370.
[82] N. Brose, A.G. Petrenko, T.C. Südhof, R. Jahn, Synaptotagmin: a calcium sensor on the synaptic vesicle surface, Science 256 (1992) 1021–1025.
[83] G. Schiavo, Q.M. Gu, G.D. Prestwich, T.H. Söllner, J.E. Rothman, Calcium-dependent switching of the specificity of phosphoinositide binding to synaptotagmin, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 13327–13332.
[84] A. Radhakrishnan, A. Stein, R. Jahn, D. Fasshauer, The Ca^{2+} affinity of synaptotagmin 1 is markedly increased by a specific interaction of its C2B domain with phosphatidylinositol 4,5-bisphosphate, J. Biol. Chem. 284 (2009) 25749–25760.
[85] M. Xue, C. Ma, T.K. Craig, C. Rosenmund, J. Rizo, The Janus-faced nature of the C2B domain is fundamental for synaptotagmin-1 function, Nat. Struct. Mol. Biol. 15 (2008) 1160–1168.
[86] R.B. Sutton, B.A. Davletov, A.M. Berghuis, T.C. Südhof, S.R. Sprang, Structure of the first C2 domain of synaptotagmin I: a novel Ca^{2+}/phospholipid-binding fold, Cell 80 (1995) 929–938.
[87] X. Shao, B.A. Davletov, R.B. Sutton, T.C. Südhof, J. Rizo, Bipartite Ca^{2+}-binding motif in C2 domains of synaptotagmin and protein kinase C, Science 273 (1996) 248–251.
[88] J.S. Rhee, L.Y. Li, O.H. Shin, J.C. Rah, J. Rizo, T.C. Südhof, C. Rosenmund, Augmenting neurotransmitter release by enhancing the apparent Ca^{2+} affinity of synaptotagmin 1, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 18664–18669.
[89] A.R. Striegel, L.M. Biela, C.S. Evans, Z. Wang, J.B. Delehoy, R.B. Sutton, E.R. Chapman, N.E. Reist, Calcium binding by synaptotagmin's C_2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission, J. Neurosci. 32 (2012) 1253–1260.
[90] J. Bai, P. Wang, E.R. Chapman, C2A activates a cryptic Ca^{2+}-triggered membrane penetration activity within the C2B domain of synaptotagmin I, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 1665–1670.
[91] E. Hui, J. Bai, E.R. Chapman, Ca^{2+}-triggered simultaneous membrane penetration of the tandem C2-domains of synaptotagmin I, Biophys. J. 91 (2006) 1767–1777.
[92] E.A. Nalefski, M.A. Wisner, J.Z. Chen, S.R. Sprang, M. Fukuda, K. Mikoshiba, J.J. Falke, C2 domains from different Ca^{2+} signaling pathways display functional and mechanistic diversity, Biochemistry 40 (2001) 3089–3100.
[93] W. Kuo, D.Z. Herrick, J.F. Ellena, D.S. Cafiso, The calcium-dependent and calcium-independent membrane binding of synaptotagmin 1: two modes of C2B binding, J. Mol. Biol. 387 (2009) 284–294.
[94] S. Martens, M.M. Kozlov, H.T. McMahon, How synaptotagmin promotes membrane fusion, Science 316 (2007) 1205–1208.
[95] H. Dai, N. Shen, D. Araç, J. Rizo, A quaternary SNARE–synaptotagmin–Ca^{2+}–phospholipid complex in neurotransmitter release, J. Mol. Biol. 367 (2007) 848–863.
[96] C. Rickman, D.A. Archer, F.A. Meunier, M. Craxton, M. Fukuda, R.D. Burgoyne, B. Davletov, Synaptotagmin interaction with the syntaxin/SNAP-25 dimer is mediated by an evolutionarily conserved motif and is sensitive to inositol hexakisphosphate, J. Biol. Chem. 279 (2004) 12574–12579.
[97] U.B. Choi, P. Strop, M. Vrljic, S. Chu, A.T. Brunger, K.R. Weninger, Single-molecule FRET-derived model of the synaptotagmin 1-SNARE fusion complex, Nat. Struct. Mol. Biol. 17 (2010) 318–324.
[98] A.L. Lai, H. Huang, D.Z. Herrick, N. Epp, D.S. Cafiso, Synaptotagmin 1 and SNAREs form a complex that is structurally heterogeneous, J. Mol. Biol. 405 (2011) 696–706.
[99] J. Tang, A. Maximov, O.H. Shin, H. Dai, J. Rizo, T.C. Südhof, A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis, Cell 126 (2006) 1175–1187.
[100] K.L. Lynch, R.R. Gerona, E.C. Larsen, R.F. Marcia, J.C. Mitchell, T.F. Martin, Synaptotagmin C2A loop 2 mediates Ca^{2+}-dependent SNARE interactions essential for Ca^{2+}-triggered vesicle exocytosis, Mol. Biol. Cell 18 (2007) 4957–4968.
[101] S.M. Young Jr., E. Neher, Synaptotagmin has an essential function in synaptic vesicle positioning for synchronous release in addition to its role as a calcium sensor, Neuron 63 (2009) 482–496.
[102] E. Hui, C.P. Johnson, J. Yao, F.M. Dunning, E.R. Chapman, Synaptotagmin-mediated bending of the target membrane is a critical step in Ca^{2+}-regulated fusion, Cell 138 (2009) 709–721.
[103] N.E. Reist, J. Buchanan, J. Li, A. DiAntonio, E.M. Buxton, T.L. Schwarz, Morphologically docked synaptic vesicles are reduced in synaptotagmin mutants of Drosophila, J. Neurosci. 18 (1998) 7662–7673.
[104] H. de Wit, A.M. Walter, I. Milosevic, A. Gulyás-Kovács, D. Riedel, J.B. Sørensen, M. Verhage, Synaptotagmin-1 docks secretory vesicles to syntaxin-1/SNAP-25 acceptor complexes, Cell 138 (2009) 935–946.
[105] M. Vrljic, P. Strop, J.A. Ernst, R.B. Sutton, S. Chu, A.T. Brunger, Molecular mechanism of the synaptotagmin–SNARE interaction in Ca^{2+}-triggered vesicle fusion, Nat. Struct. Mol. Biol. 17 (2010) 325–331.
[106] A. Stein, A. Radhakrishnan, D. Riedel, D. Fasshauer, R. Jahn, Synaptotagmin activates membrane fusion through a Ca^{2+}-dependent *trans* interaction with phospholipids, Nat. Struct. Mol. Biol. 14 (2007) 904–911.
[107] X. Chen, D. Araç, T.M. Wang, C.J. Gilpin, J. Zimmerberg, J. Rizo, SNARE-mediated lipid mixing depends on the physical state of the vesicles, Biophys. J. 90 (2006) 2062–2074.
[108] M.K. Domanska, V. Kiessling, A. Stein, D. Fasshauer, L.K. Tamm, Single vesicle millisecond fusion kinetics reveals number of SNARE complexes optimal for fast SNARE-mediated membrane fusion, J. Biol. Chem. 284 (2009) 32158–32166.
[109] G. van den Bogaart, M.G. Holt, G. Bunt, D. Riedel, F.S. Wouters, R. Jahn, One SNARE complex is sufficient for membrane fusion, Nat. Struct. Mol. Biol. 17 (2010) 358–364.
[110] S. Schoch, E.D. Gundelfinger, Molecular organization of the presynaptic active zone, Cell Tissue Res. 326 (2006) 379–391.
[111] C.C. Garner, S. Kindler, E.D. Gundelfinger, Molecular determinants of presynaptic active zones, Curr. Opin. Neurobiol. 10 (2000) 321–327.
[112] D.E. Chandler, J.E. Heuser, Arrest of membrane fusion events in mast cells by quick-freezing, J. Cell Biol. 86 (1980) 666–674.
[113] E. Hui, J.D. Gaffaney, Z. Wang, C.P. Johnson, C.S. Evans, E.R. Chapman, Mechanism and function of synaptotagmin-mediated membrane apposition, Nat. Struct. Mol. Biol. 18 (2011) 813–821.
[114] J.A. Killian, Hydrophobic mismatch between proteins and lipids in membranes, Biochim. Biophys. Acta 1376 (1998) 401–415.
[115] R. Mohrmann, H. de Wit, M. Verhage, E. Neher, J.B. Sørensen, Fast vesicle fusion in living cells requires at least three SNARE complexes, Science 330 (2010) 502–505.
[116] R. Sinha, S. Ahmed, R. Jahn, J. Klingauf, Two synaptobrevin molecules are sufficient for vesicle fusion in central nervous system synapses, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 14318–14323.
[117] S.O. Rizzoli, R. Jahn, 'Kiss-and-run', collapse and 'readily retrievable' vesicles, Traffic 8 (2007) 1137–1144.
[118] B. Ceccarelli, W.P. Hurlbut, A. Mauro, Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction, J. Cell Biol. 57 (1973) 499–524.
[119] A.M. Aravanis, J.L. Pyle, R.W. Tsien, Single synaptic vesicles fusing transiently and successively without loss of identity, Nature 423 (2003) 643–647.
[120] T.C. Südhof, The synaptic vesicle cycle, Annu. Rev. Neurosci. 27 (2004) 509–547.
[121] J.E. Heuser, T.S. Reese, Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction, J. Cell Biol. 57 (1973) 315–344.
[122] B.M. Pearse, Coated vesicles from pig brain: purification and biochemical characterization, J. Mol. Biol. 97 (1975) 93–98.
[123] P.R. Maycox, E. Link, A. Reetz, S.A. Morris, R. Jahn, Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling, J. Cell Biol. 118 (1992) 1379–1388.
[124] B. Granseth, B. Odermatt, S.J. Royle, L. Lagnado, Clathrin-mediated endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses, Neuron 51 (2006) 773–786.
[125] V.N. Murthy, C.F. Stevens, Synaptic vesicles retain their identity through the endocytic cycle, Nature 392 (1998) 497–501.
[126] P. Hoopmann, A. Punge, S.V. Barysch, V. Westphal, J. Bückers, F. Opazo, I. Bethani, M.A. Lauterbach, S.W. Hell, S.O. Rizzoli, Endosomal sorting of readily releasable synaptic vesicles, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 19055–19060.
[127] R. Jahn, T.C. Südhof, Synaptic vesicle traffic: rush hour in the nerve terminal, J. Neurochem. 61 (1993) 12–21.
[128] Q. Zhang, Y. Li, R.W. Tsien, The dynamic control of 'kiss-and-run' and vesicular reuse probed with single nanoparticles, Science 323 (2009) 1448–1453.

[129] W.J. Jockusch, G.J. Praefcke, H.T. McMahon, L. Lagnado, Clathrin-dependent and clathrin-independent retrieval of synaptic vesicles in retinal bipolar cells, Neuron 46 (2005) 869–878.
[130] P. Verstreken, O. Kjaerulff, T.E. Lloyd, R. Atkinson, Y. Zhou, I.A. Meinertzhagen, H.J. Bellen, Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release, Cell 109 (2002) 101–112.
[131] S. Höning, D. Ricotta, M. Krauss, K. Späte, B. Spolaore, A. Motley, M. Robinson, C. Robinson, V. Haucke, D.J. Owen, Phosphatidylinositol-4,5-bisphosphate regulates sorting signal recognition by the clathrin-associated adaptor complex AP2, Mol. Cell 18 (2005) 519–531.
[132] L.P. Jackson, B.T. Kelly, A.J. McCoy, T. Gaffry, L.C. James, B.M. Collins, S. Höning, P.R. Evans, D.J. Owen, A large-scale conformational change couples membrane recruitment to cargo binding in the AP2 clathrin adaptor complex, Cell 141 (2010) 1220–1229.
[133] E.M. Jorgensen, E. Hartwieg, K. Schuske, M.L. Nonet, Y. Jin, H.R. Horvitz, Defective recycling of synaptic vesicles in synaptotagmin mutants of *Caenorhabditis elegans*, Nature 378 (1995) 196–199.
[134] E.M. Schmid, H.T. McMahon, Integrating molecular and network biology to decode endocytosis, Nature 448 (2007) 883–888.
[135] M. Mettlen, D. Loerke, D. Yarar, G. Danuser, S.L. Schmid, Cargo- and adaptor-specific mechanisms regulate clathrin-mediated endocytosis, J. Cell Biol. 188 (2010) 919–933.
[136] D. Nunez, C. Antonescu, M. Mettlen, A. Liu, S.L. Schmid, D. Loerke, G. Danuser, Hotspots organize clathrin-mediated endocytosis by efficient recruitment and retention of nucleating resources, Traffic 12 (2011) 1868–1878.
[137] B.M. Pearse, C.J. Smith, D.J. Owen, Clathrin coat construction in endocytosis, Curr. Opin. Struct. Biol. 10 (2000) 220–228.
[138] C.N. Antonescu, F. Aguet, G. Danuser, S.L. Schmid, Phosphatidylinositol-4,5-bisphosphate regulates clathrin-coated pit initiation, stabilization, and size, Mol. Biol. Cell 22 (2011) 2588–2600.
[139] M. Krauss, V. Kukhtina, A. Pechstein, V. Haucke, Stimulation of phosphatidylinositol kinase type I-mediated phosphatidylinositol 4,5-bisphosphate synthesis by AP-2μ-cargo complexes, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 11934–11939.
[140] S.M. Voglmaier, K. Kam, H. Yang, D.L. Fortin, Z. Hua, R.A. Nicoll, R.H. Edwards, Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling, Neuron 51 (2006) 71–84.
[141] P.K. Tan, C. Waites, Y. Liu, D.E. Krantz, R.H. Edwards, A leucine-based motif mediates the endocytosis of vesicular monoamine and acetylcholine transporters, J. Biol. Chem. 273 (1998) 17351–17360.
[142] V. Haucke, P. De Camilli, AP2 recruitment to synaptotagmin stimulated by tyrosine-based endocytic motifs, Science 285 (1999) 1268–1271.
[143] J.Z. Zhang, B.A. Davletov, T.C. Südhof, R.G. Anderson, Synaptotagmin I is a high affinity receptor for clathrin AP2: implications for membrane recycling, Cell 78 (1994) 751–760.
[144] J. Yao, A. Nowack, P. Kensel-Hammes, R.G. Gardner, S.M. Bajjalieh, Cotrafficking of SV2 and synaptotagmin at the synapse, J. Neurosci. 30 (2010) 5569–5578.
[145] M.B. Feany, A.G. Yee, M.L. Delvy, K.M. Buckley, The synaptic vesicle proteins SV2, synaptotagmin and synaptophysin are sorted to separate cellular compartments in CHO fibroblasts, J. Cell Biol. 123 (1993) 575–584.
[146] N. Jarousse, R.B. Kelly, The AP2 binding site of synaptotagmin 1 is not an internalization signal but a regulator of endocytosis, J. Cell Biol. 154 (2001) 857–866.
[147] D.T. Stimson, P.S. Estes, S. Rao, K.S. Krishnan, L.E. Kelly, M. Ramaswami, *Drosophila* stoned proteins regulate the rate and fidelity of synaptic vesicle internalization, J. Neurosci. 21 (2001) 3034–3044.
[148] S. Harada, I. Hori, H. Yamamoto, R. Hosono, Mutations in the unc-41 gene cause elevation of acetylcholine levels, J. Neurochem. 63 (1994) 439–446.
[149] K. Walther, M. Krauss, M.K. Diril, S. Lemke, D. Ricotta, S. Höning, S. Kaiser, V. Haucke, Human stoned B interacts with AP2 and synaptotagmin and facilitates clathrin-coated vesicle uncoating, EMBO Rep. 2 (2001) 634–640.
[150] K. Walther, M.K. Diril, N. Jung, V. Haucke, Functional dissection of the interactions of stonin 2 with the adaptor complex AP2 and synaptotagmin, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 964–969.
[151] N. Jung, M. Wienisch, M. Gu, J.B. Rand, S.L. Müller, G. Krause, E.M. Jorgensen, J. Klingauf, V. Haucke, Molecular basis of synaptic vesicle cargo recognition by the endocytic sorting adaptor stonin 2, J. Cell Biol. 179 (2007) 1497–1510.
[152] J.A. Martina, C.J. Bonangelino, R.C. Aguilar, J.S. Bonifacino, Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery, J. Cell Biol. 153 (2001) 1111–1120.
[153] S.H. Kim, T.A. Ryan, Synaptic vesicle recycling at CNS snapses without AP2, J. Neurosci. 29 (2009) 3865–3874.
[154] E. Boucrot, S. Saffarian, R. Zhang, T. Kirchhausen, Roles of AP2 in clathrin-mediated endocytosis, PLoS One 5 (2010) e10597.
[155] T.A. Ryan, Presynaptic imaging techniques, Curr. Opin. Neurobiol. 11 (2001) 544–549.
[156] J. Yao, S.E. Kwon, J.D. Gaffaney, F.M. Dunning, E.R. Chapman, Uncoupling the roles of synaptotagmin I during endo- and exocytosis of synaptic vesicles, Nat. Neurosci. 15 (2011) 243–249.
[157] G. Miesenböck, D.A. De Angelis, J.E. Rothman, Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins, Nature 394 (1998) 192–195.
[158] K.E. Poskanzer, R.D. Fetter, G.W. Davis, Discrete residues in the C2B domain of synaptotagmin I independently specify endocytic rate and synaptic vesicle size, Neuron 50 (2006) 49–62.
[159] R. Mohrmann, H.J. Matthies, E. Woodruff III, K. Broadie, Stoned B mediates sorting of integral synaptic vesicle proteins, Neuroscience 153 (2008) 1048–1063.
[160] A.E. Schivell, R.H. Batchelor, S.M. Bajjalieh, Isoform-specific, calcium-regulated interaction of the synaptic vesicle proteins SV2 and synaptotagmin, J. Biol. Chem. 271 (1996) 27770–27775.
[161] E. Hui, J. Bai, P. Wang, M. Sugimori, R.R. Llinas, E.R. Chapman, Three distinct kinetic groupings of the synaptotagmin family: candidate sensors for rapid and delayed exocytosis, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 5210–5214.
[162] C.R. Artalejo, J.R. Henley, M.A. McNiven, H.C. Palfrey, Rapid endocytosis coupled to exocytosis in adrenal chromaffin cells involves Ca^{2+}, GTP, and dynamin but not clathrin, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 8328–8332.
[163] C.R. Artalejo, A. Elhamdani, H.C. Palfrey, Calmodulin is the divalent cation receptor for rapid endocytosis, but not exocytosis, in adrenal chromaffin cells, Neuron 16 (1996) 195–205.
[164] R.A. Pyle, A.E. Schivell, H. Hidaka, S.M. Bajjalieh, Phosphorylation of synaptic vesicle protein 2 modulates binding to synaptotagmin, J. Biol. Chem. 275 (2000) 17195–17200.
[165] H. Kuromi, M. Yoshihara, Y. Kidokoro, An inhibitory role of calcineurin in endocytosis of synaptic vesicles at nerve terminals of *Drosophila* larvae, Neurosci. Res. 27 (1997) 101–113.
[166] M.K. Bennett, N. Calakos, T. Kreiner, R.H. Scheller, Synaptic vesicle membrane proteins interact to form a multimeric complex, J. Cell Biol. 116 (1992) 761–775.
[167] J.Y. Jia, S. Lamer, M. Schümann, M.R. Schmidt, E. Krause, V. Haucke, Quantitative proteomics analysis of detergent-resistant membranes from chemical synapses: evidence for cholesterol as spatial organizer of synaptic vesicle cycling, Mol. Cell. Proteomics 5 (2006) 2060–2071.
[168] K.I. Willig, S.O. Rizzoli, V. Westphal, R. Jahn, S.W. Hell, STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis, Nature 440 (2006) 935–939.
[169] F. Opazo, A. Punge, J. Bückers, P. Hoopmann, L. Kastrup, S.W. Hell, S.O. Rizzoli, Limited intermixing of synaptic vesicle components upon vesicle recycling, Traffic 11 (2010) 800–812.
[170] S.J. Koo, S. Markovic, D. Puchkov, C.C. Mahrenholz, F. Beceren-Braun, T. Maritzen, J. Dernedde, R. Volkmer, H. Oschkinat, V. Haucke, SNARE motif-mediated sorting of synaptobrevin by the endocytic adaptors clathrin assembly lymphoid myeloid leukemia (CALM) and AP180 at synapses, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 13540–13545.
[171] F. Deák, S. Schoch, X. Liu, T.C. Südhof, E.T. Kavalali, Synaptobrevin is essential for fast synaptic vesicle endocytosis, Nat. Cell Biol. 6 (2004) 1102–1108.
[172] T.W. Groemer, C.S. Thiel, M. Holt, D. Riedel, Y. Hua, J. Hüve, B.G. Wilhelm, J. Klingauf, Amyloid precursor protein is trafficked and secreted via synaptic vesicles, PLoS One 6 (2011) e18754.
[173] S.E. Kwon, E.R. Chapman, Synaptophysin regulates the kinetics of synaptic vesicle endocytosis in central neurons, Neuron 70 (2011) 847–854.
[174] T.E. Lloyd, P. Verstreken, E.J. Ostrin, A. Phillippi, O. Lichtarge, H.J. Bellen, A genome-wide search for synaptic vesicle cycle proteins in *Drosophila*, Neuron 26 (2000) 45–50.
[175] S.A. Mutch, P. Kensel-Hammes, J.C. Gadd, B.S. Fujimoto, R.W. Allen, P.G. Schiro, R.M. Lorenz, C.L. Kuyper, J.S. Kuo, S.M. Bajjalieh, D.T. Chiu, Protein quantification at the single vesicle level reveals that a subset of synaptic vesicle proteins are trafficked with high precision, J. Neurosci. 31 (2011) 1461–1470.
[176] J.S. Dittman, J.M. Kaplan, Factors regulating the abundance and localization of synaptobrevin in the plasma membrane, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 11399–11404.
[177] T. Fernández-Alfonso, R. Kwan, T.A. Ryan, Synaptic vesicles interchange their membrane proteins with a large surface reservoir during recycling, Neuron 51 (2006) 179–186.
[178] M. Wienisch, J. Klingauf, Vesicular proteins exocytosed and subsequently retrieved by compensatory endocytosis are nonidentical, Nat. Neurosci. 9 (2006) 1019–1027.
[179] V. Haucke, E. Neher, S.J. Sigrist, Protein scaffolds in the coupling of synaptic exocytosis and endocytosis, Nat. Rev. Neurosci. 12 (2011) 127–138.
[180] S.M. Voglmaier, R.H. Edwards, Do different endocytic pathways make different synaptic vesicles? Curr. Opin. Neurobiol. 17 (2007) 374–380.
[181] C. Eggeling, C. Ringemann, R. Medda, G. Schwarzmann, K. Sandhoff, S. Polyakova, V.N. Belov, B. Hein, C. von Middendorff, A. Schönle, S.W. Hell, Direct observation of the nanoscale dynamics of membrane lipids in a living cell, Nature 457 (2009) 1159–1162.
[182] S.J. Sahl, M. Leutenegger, M. Hilbert, S.W. Hell, C. Eggeling, Fast molecular tracking maps nanoscale dynamics of plasma membrane lipids, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 6829–6834.
[183] R.M. Perera, R. Zoncu, L. Lucast, P. De Camilli, D. Toomre, Two synaptojanin 1 isoforms are recruited to clathrin-coated pits at different stages, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 19332–19337.
[184] N. Ringstad, Y. Nemoto, P. De Camilli, The SH3p4/Sh3p8/SH3p13 protein family: binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 8569–8574.
[185] J.L. Gallop, C.C. Jao, H.M. Kent, P.J. Butler, P.R. Evans, R. Langen, H.T. McMahon, Mechanism of endophilin N-BAR domain-mediated membrane curvature, EMBO J. 25 (2006) 2898–2910.
[186] K. Farsad, N. Ringstad, K. Takei, S.R. Floyd, K. Rose, P. De Camilli, Generation of high curvature membranes mediated by direct endophilin bilayer interactions, J. Cell Biol. 155 (2001) 193–200.
[187] B.J. Peter, H.M. Kent, I.G. Mills, Y. Vallis, P.J. Butler, P.R. Evans, H.T. McMahon, BAR domains as sensors of membrane curvature: the amphiphysin BAR structure, Science 303 (2004) 495–499.
[188] B. Chang-Ileto, S.G. Frere, R.B. Chan, S.V. Voronov, A. Roux, G. Di Paolo, Synaptojanin 1-mediated PIP_2 hydrolysis is modulated by membrane curvature and facilitates membrane fission, Dev. Cell 20 (2011) 206–218.

[189] I. Milosevic, S. Giovedi, X. Lou, A. Raimondi, C. Collesi, H. Shen, S. Paradise, E. O'Toole, S. Ferguson, O. Cremona, P.D. Camilli, Recruitment of endophilin to clathrin-coated pit necks is required for efficient vesicle uncoating after fission, Neuron 72 (2011) 587–601.
[190] S.Y. Lee, M.R. Wenk, Y. Kim, A.C. Nairn, P. De Camilli, Regulation of synaptojanin 1 by cyclin-dependent kinase 5 at synapses, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 546–551.
[191] F. Darios, E. Connell, B. Davletov, Phospholipases and fatty acid signalling in exocytosis, J. Physiol. 585 (2007) 699–704.
[192] B. Davletov, E. Connell, F. Darios, Regulation of SNARE fusion machinery by fatty acids, Cell. Mol. Life Sci. 64 (2007) 1597–1608.
[193] B. Davletov, C. Montecucco, Lipid function at synapses, Curr. Opin. Neurobiol. 20 (2010) 543–549.
[194] J. Rohrbough, K. Broadie, Lipid regulation of the synaptic vesicle cycle, Nat. Rev. Neurosci. 6 (2005) 139–150.
[195] D. Piomelli, G. Astarita, R. Rapaka, A neuroscientist's guide to lipidomics, Nat. Rev. Neurosci. 8 (2007) 743–754.
[196] C. Schultz, A.B. Neef, T.W. Gadella Jr., J. Goedhart, Imaging lipids in living cells, Cold Spring Harb. Protoc. 2010 (2010) pdb top83.
[197] J.A. Prescher, C.R. Bertozzi, Chemistry in living systems, Nat. Chem. Biol. 1 (2005) 13–21.
[198] A.B. Neef, C. Schultz, Selective fluorescence labeling of lipids in living cells, Angew. Chem. Int. Ed Engl. 48 (2009) 1498–1500.
[199] C.Y. Jao, M. Roth, R. Welti, A. Salic, Metabolic labeling and direct imaging of choline phospholipids *in vivo*, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 15332–15337.
[200] N.J. Agard, J.A. Prescher, C.R. Bertozzi, A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems, J. Am. Chem. Soc. 126 (2004) 15046–15047.
[201] M. Mentel, V. Laketa, D. Subramanian, H. Gillandt, C. Schultz, Photoactivatable and cell-membrane-permeable phosphatidylinositol 3,4,5-trisphosphate, Angew. Chem. Int. Ed Engl. 50 (2011) 3811–3814.
[202] D.M. Spencer, T.J. Wandless, S.L. Schreiber, G.R. Crabtree, Controlling signal transduction with synthetic ligands, Science 262 (1993) 1019–1024.
[203] T. Inoue, W.D. Heo, J.S. Grimley, T.J. Wandless, T. Meyer, An inducible translocation strategy to rapidly activate and inhibit small GTPase signaling pathways, Nat. Methods 2 (2005) 415–418.
[204] P.J. Belshaw, S.N. Ho, G.R. Crabtree, S.L. Schreiber, Controlling protein association and subcellular localization with a synthetic ligand that induces heterodimerization of proteins, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 4604–4607.
[205] B.C. Suh, T. Inoue, T. Meyer, B. Hille, Rapid chemically induced changes of PtdIns(4,5)P_2 gate KCNQ ion channels, Science 314 (2006) 1454–1457.
[206] P. Varnai, B. Thyagarajan, T. Rohacs, T. Balla, Rapidly inducible changes in phosphatidylinositol 4,5-bisphosphate levels influence multiple regulatory functions of the lipid in intact living cells, J. Cell Biol. 175 (2006) 377–382.
[207] R. Zoncu, R.M. Perera, R. Sebastian, F. Nakatsu, H. Chen, T. Balla, G. Ayala, D. Toomre, P.V. De Camilli, Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 3793–3798.
[208] C.e.S. Consortium, Genome sequence of the nematode *C. elegans*: a platform for investigating biology, Science 282 (1998) 2012–2018.
[209] M.D. Adams, S.E. Celniker, R.A. Holt, C.A. Evans, J.D. Gocayne, P.G. Amanatides, S.E. Scherer, P.W. Li, R.A. Hoskins, R.F. Galle, R.A. George, S.E. Lewis, S. Richards, M. Ashburner, S.N. Henderson, G.G. Sutton, J.R. Wortman, M.D. Yandell, Q. Zhang, L.X. Chen, R.C. Brandon, Y.H. Rogers, R.G. Blazej, M. Champe, B.D. Pfeiffer, K.H. Wan, C. Doyle, E.G. Baxter, G. Helt, C.R. Nelson, G.L. Gabor, J.F. Abril, A. Agbayani, H.J. An, C. Andrews-Pfannkoch, D. Baldwin, R.M. Ballew, A. Basu, J. Baxendale, L. Bayraktaroglu, E.M. Beasley, K.Y. Beeson, P.V. Benos, B.P. Berman, D. Bhandari, S. Bolshakov, D. Borkova, M.R. Botchan, J. Bouck, P. Brokstein, P. Brottier, K.C. Burtis, D.A. Busam, H. Butler, E. Cadieu, A. Center, I. Chandra, J.M. Cherry, S. Cawley, C. Dahlke, L.B. Davenport, P. Davies, B. de Pablos, A. Delcher, Z. Deng, A.D. Mays, I. Dew, S.M. Dietz, K. Dodson, L.E. Doup, M. Downes, S. Dugan-Rocha, B.C. Dunkov, P. Dunn, K.J. Durbin, C.C. Evangelista, C. Ferraz, S. Ferriera, W. Fleischmann, C. Fosler, A.E. Gabrielian, N.S. Garg, W.M. Gelbart, K. Glasser, A. Glodek, F. Gong, J.H. Gorrell, Z. Gu, P. Guan, M. Harris, N.L. Harris, D. Harvey, T.J. Heiman, J.R. Hernandez, J. Houck, D. Hostin, K.A. Houston, T.J. Howland, M.H. Wei, C. Ibegwam, M. Jalali, F. Kalush, G.H. Karpen, Z. Ke, J.A. Kennison, K.A. Ketchum, B.E. Kimmel, C.D. Kodira, C. Kraft, S. Kravitz, D. Kulp, Z. Lai, P. Lasko, Y. Lei, A.A. Levitsky, J. Li, Z. Li, Y. Liang, X. Lin, X. Liu, B. Mattei, T.C. McIntosh, M.P. McLeod, D. McPherson, G. Merkulov, N.V. Milshina, C. Mobarry, J. Morris, A. Moshrefi, S.M. Mount, M. Moy, B. Murphy, L. Murphy, D.M. Muzny, D.L. Nelson, D.R. Nelson, K.A. Nelson, K. Nixon, D.R. Nusskern, J.M. Pacleb, M. Palazzolo, G.S. Pittman, S. Pan, J. Pollard, V. Puri, M.G. Reese, K. Reinert, K. Remington, R.D. Saunders, F. Scheeler, H. Shen, B.C. Shue, I. Sidén-Kiamos, M. Simpson, M.P. Skupski, T. Smith, E. Spier, A.C. Spradling, M. Stapleton, R. Strong, E. Sun, R. Svirskas, C. Tector, R. Turner, E. Venter, A.H. Wang, X. Wang, Z.Y. Wang, D.A. Wassarman, G.M. Weinstock, J. Weissenbach, S.M. Williams, T. Woodage, K.C. Worley, D. Wu, S. Yang, Q.A. Yao, J. Ye, R.F. Yeh, J.S. Zaveri, M. Zhan, G. Zhang, Q. Zhao, L. Zheng, X.H. Zheng, F.N. Zhong, W. Zhong, X. Zhou, S. Zhu, X. Zhu, H.O. Smith, R.A. Gibbs, E.W. Myers, G.M. Rubin, J.C. Venter, The genome sequence of *Drosophila melanogaster*, Science 287 (2000) 2185–2195.
[210] R.H. Waterston, K. Lindblad-Toh, E. Birney, J. Rogers, J.F. Abril, P. Agarwal, R. Agarwala, R. Ainscough, M. Alexandersson, P. An, S.E. Antonarakis, J. Attwood, R. Baertsch, J. Bailey, K. Barlow, S. Beck, E. Berry, B. Birren, T. Bloom, P. Bork, M. Botcherby, N. Bray, M.R. Brent, D.G. Brown, S.D. Brown, C. Bult, J. Burton, J. Butler, R.D. Campbell, P. Carninci, S. Cawley, F. Chiaromonte, A.T. Chinwalla, D.M. Church, M. Clamp, C. Clee, F.S. Collins, L.L. Cook, R.R. Copley, A. Coulson, O. Couronne, J. Cuff, V. Curwen, T. Cutts, M. Daly, R. David, J. Davies, K.D. Delehaunty, J. Deri, E.T. Dermitzakis, C. Dewey, N.J. Dickens, M. Diekhans, S. Dodge, I. Dubchak, D.M. Dunn, S.R. Eddy, L. Elnitski, R.D. Emes, P. Eswara, E. Eyras, A. Felsenfeld, G.A. Fewell, P. Flicek, K. Foley, W.N. Frankel, L.A. Fulton, R.S. Fulton, T.S. Furey, D. Gage, R.A. Gibbs, G. Glusman, S. Gnerre, N. Goldman, L. Goodstadt, D. Grafham, T.A. Graves, E.D. Green, S. Gregory, R. Guigó, M. Guyer, R.C. Hardison, D. Haussler, Y. Hayashizaki, L.W. Hillier, A. Hinrichs, W. Hlavina, T. Holzer, F. Hsu, A. Hua, T. Hubbard, A. Hunt, I. Jackson, D.B. Jaffe, L.S. Johnson, M. Jones, T.A. Jones, A. Joy, M. Kamal, E.K. Karlsson, D. Karolchik, A. Kasprzyk, J. Kawai, E. Keibler, C. Kells, W.J. Kent, A. Kirby, D.L. Kolbe, I. Korf, R.S. Kucherlapati, E.J. Kulbokas, D. Kulp, T. Landers, J.P. Leger, S. Leonard, I. Letunic, R. Levine, J. Li, M. Li, C. Lloyd, S. Lucas, B. Ma, D.R. Maglott, E.R. Mardis, L. Matthews, E. Mauceli, J.H. Mayer, M. McCarthy, W.R. McCombie, S. McLaren, K. McLay, J.D. McPherson, J. Meldrim, B. Meredith, J.P. Mesirov, W. Miller, T.L. Miner, E. Mongin, K.T. Montgomery, M. Morgan, R. Mott, J.C. Mullikin, D.M. Muzny, W.E. Nash, J.O. Nelson, M.N. Nhan, R. Nicol, Z. Ning, C. Nusbaum, M.J. O'Connor, Y. Okazaki, K. Oliver, E. Overton-Larty, L. Pachter, G. Parra, K.H. Pepin, J. Peterson, P. Pevzner, R. Plumb, C.S. Pohl, A. Poliakov, T.C. Ponce, C.P. Ponting, S. Potter, M. Quail, A. Reymond, B.A. Roe, K.M. Roskin, E.M. Rubin, A.G. Rust, R. Santos, V. Sapojnikov, B. Schultz, J. Schultz, M.S. Schwartz, S. Schwartz, C. Scott, S. Seaman, S. Searle, T. Sharpe, A. Sheridan, R. Shownkeen, S. Sims, J.B. Singer, G. Slater, A. Smit, D.R. Smith, B. Spencer, A. Stabenau, N. Stange-Thomann, C. Sugnet, M. Suyama, G. Tesler, J. Thompson, D. Torrents, E. Trevaskis, J. Tromp, C. Ucla, A. Ureta-Vidal, J.P. Vinson, A.C. Von Niederhausern, C.M. Wade, M. Wall, R.J. Weber, R.B. Weiss, M.C. Wendl, A.P. West, K. Wetterstrand, R. Wheeler, S. Whelan, J. Wierzbowski, D. Willey, S. Williams, R.K. Wilson, E. Winter, K.C. Worley, D. Wyman, S. Yang, S.P. Yang, E.M. Zdobnov, M.C. Zody, E.S. Lander, Initial sequencing and comparative analysis of the mouse genome, Nature 420 (2002) 520–562.
[211] E. Marza, T. Long, A. Saiardi, M. Sumakovic, S. Eimer, D.H. Hall, G.M. Lesa, Polyunsaturated fatty acids influence synaptojanin localization to regulate synaptic vesicle recycling, Mol. Biol. Cell 19 (2008) 833–842.
[212] F.D. Huang, H.J. Matthies, S.D. Speese, M.A. Smith, K. Broadie, Rolling blackout, a newly identified PIP_2-DAG pathway lipase required for *Drosophila* phototransduction, Nat. Neurosci. 7 (2004) 1070–1078.
[213] P.T. Ivanova, S.B. Milne, J.S. Forrester, H.A. Brown, LIPID arrays: new tools in the understanding of membrane dynamics and lipid signaling, Mol. Interv. 4 (2004) 86–96.
[214] G.M. Rubin, M.D. Yandell, J.R. Wortman, G.L. Gabor Miklos, C.R. Nelson, I.K. Hariharan, M.E. Fortini, P.W. Li, R. Apweiler, W. Fleischmann, J.M. Cherry, S. Henikoff, M.P. Skupski, S. Misra, M. Ashburner, E. Birney, M.S. Boguski, T. Brody, P. Brokstein, S.E. Celniker, S.A. Chervitz, D. Coates, A. Cravchik, A. Gabrielian, R.F. Galle, W.M. Gelbart, R.A. George, L.S. Goldstein, F. Gong, P. Guan, N.L. Harris, B.A. Hay, R.A. Hoskins, J. Li, Z. Li, R.O. Hynes, S.J. Jones, P.M. Kuehl, B. Lemaitre, J.T. Littleton, D.K. Morrison, C. Mungall, P.H. O'Farrell, O.K. Pickeral, C. Shue, L.B. Vosshall, J. Zhang, Q. Zhao, X.H. Zheng, S. Lewis, Comparative genomics of the eukaryotes, Science 287 (2000) 2204–2215.
[215] D.K. Morrison, M.S. Murakami, V. Cleghon, Protein kinases and phosphatases in the *Drosophila* genome, J. Cell Biol. 150 (2000) F57–F62.
[216] F. Christiansen, C. Zube, T.F. Andlauer, C. Wichmann, W. Fouquet, D. Owald, S. Mertel, F. Leiss, G. Tavosanis, A.J. Luna, A. Fiala, S.J. Sigrist, Presynapses in Kenyon cell dendrites in the mushroom body calyx of *Drosophila*, J. Neurosci. 31 (2011) 9696–9707.
[217] L.M. Masuda-Nakagawa, N.K. Tanaka, C.J. O'Kane, Stereotypic and random patterns of connectivity in the larval mushroom body calyx of *Drosophila*, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 19027–19032.
[218] V. Uytterhoeven, S. Kuenen, J. Kasprowicz, K. Miskiewicz, P. Verstreken, Loss of skywalker reveals synaptic endosomes as sorting stations for synaptic vesicle proteins, Cell 145 (2011) 117–132.
[219] B. Ataman, J. Ashley, M. Gorczyca, P. Ramachandran, W. Fouquet, S.J. Sigrist, V. Budnik, Rapid activity-dependent modifications in synaptic structure and function require bidirectional Wnt signaling, Neuron 57 (2008) 705–718.
[220] R. Niwa, Y.S. Niwa, The fruit fly *Drosophila melanogaster* as a model system to study cholesterol metabolism and homeostasis, Cholesterol 2011 (2011) 176802.
[221] G. Dietzl, D. Chen, F. Schnorrer, K.C. Su, Y. Barinova, M. Fellner, B. Gasser, K. Kinsey, S. Oppel, S. Scheiblauer, A. Couto, V. Marra, K. Keleman, B.J. Dickson, A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*, Nature 448 (2007) 151–156.
[222] J.Q. Ni, L.P. Liu, R. Binari, R. Hardy, H.S. Shim, A. Cavallaro, M. Booker, B.D. Pfeiffer, M. Markstein, H. Wang, C. Villalta, T.R. Laverty, L.A. Perkins, N. Perrimon, A *Drosophila* resource of transgenic RNAi lines for neurogenetics, Genetics 182 (2009) 1089–1100.
[223] A.H. Brand, N. Perrimon, Targeted gene expression as a means of altering cell fates and generating dominant phenotypes, Development 118 (1993) 401–415.
[224] B.D. Pfeiffer, T.T. Ngo, K.L. Hibbard, C. Murphy, A. Jenett, J.W. Truman, G.M. Rubin, Refinement of tools for targeted gene expression in *Drosophila*, Genetics 186 (2010) 735–755.
[225] T. Lee, L. Luo, Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis, Neuron 22 (1999) 451–461.
[226] C.J. Potter, B. Tasic, E.V. Russler, L. Liang, L. Luo, The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis, Cell 141 (2010) 536–548.
[227] C.Y. Ting, S. Gu, S. Guttikonda, T.Y. Lin, B.H. White, C.H. Lee, Focusing transgene expression in *Drosophila* by coupling Gal4 with a novel split-LexA expression system, Genetics 188 (2011) 229–233.

[228] H.J. Bellen, R.W. Levis, G. Liao, Y. He, J.W. Carlson, G. Tsang, M. Evans-Holm, P.R. Hiesinger, K.L. Schulze, G.M. Rubin, R.A. Hoskins, A.C. Spradling, The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes, Genetics 167 (2004) 761–781.
[229] G.M. Rubin, A.C. Spradling, Genetic transformation of *Drosophila* with transposable element vectors, Science 218 (1982) 348–353.
[230] B.A. Hassan, S.N. Prokopenko, S. Breuer, B. Zhang, A. Paululat, H.J. Bellen, Skittles, a *Drosophila* phosphatidylinositol 4-phosphate 5-kinase, is required for cell viability, germline development and bristle morphology, but not for neurotransmitter release, Genetics 150 (1998) 1527–1537.
[231] Y.S. Rong, K.G. Golic, Gene targeting by homologous recombination in *Drosophila*, Science 288 (2000) 2013–2018.
[232] C.M. Choi, S. Vilain, M. Langen, S. Van Kelst, N. De Geest, J. Yan, P. Verstreken, B.A. Hassan, Conditional mutagenesis in *Drosophila*, Science 324 (2009) 54.
[233] G. Gao, C. McMahon, J. Chen, Y.S. Rong, A powerful method combining homologous recombination and site-specific recombination for targeted mutagenesis in *Drosophila*, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 13999–14004.
[234] H. Heerssen, R.D. Fetter, G.W. Davis, Clathrin dependence of synaptic-vesicle formation at the *Drosophila* neuromuscular junction, Curr. Biol. 18 (2008) 401–409.
[235] J. Kasprowicz, S. Kuenen, K. Miskiewicz, R.L. Habets, L. Smitz, P. Verstreken, Inactivation of clathrin heavy chain inhibits synaptic recycling but allows bulk membrane uptake, J. Cell Biol. 182 (2008) 1007–1016.
[236] K.W. Marek, G.W. Davis, Transgenically encoded protein photoinactivation (FlAsH-FALI): acute inactivation of synaptotagmin I, Neuron 36 (2002) 805–813.
[237] I. Kessell, B.D. Holst, T.F. Roth, Membranous intermediates in endocytosis are labile, as shown in a temperature-sensitive mutant, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 4968–4972.
[238] E.D. Gundelfinger, M.M. Kessels, B. Qualmann, Temporal and spatial coordination of exocytosis and endocytosis, Nat. Rev. Mol. Cell Biol. 4 (2003) 127–139.
[239] S.M. Wojcik, N. Brose, Regulation of membrane fusion in synaptic excitation-secretion coupling: speed and accuracy matter, Neuron 55 (2007) 11–24.
[240] B. Katz, R. Miledi, Ionic requirements of synaptic transmitter release, Nature 215 (1967) 651.
[241] J. Roos, R.B. Kelly, The endocytic machinery in nerve terminals surrounds sites of exocytosis, Curr. Biol. 9 (1999) 1411–1414.
[242] P.P. Atluri, T.A. Ryan, The kinetics of synaptic vesicle reacidification at hippocampal nerve terminals, J. Neurosci. 26 (2006) 2313–2320.
[243] P.R. Maycox, T. Deckwerth, J.W. Hell, R. Jahn, Glutamate uptake by brain synaptic vesicles. Energy dependence of transport and functional reconstitution in proteoliposomes, J. Biol. Chem. 263 (1988) 15423–15428.
[244] J.W. Hell, P.R. Maycox, H. Stadler, R. Jahn, Uptake of GABA by rat brain synaptic vesicles isolated by a new procedure, EMBO J. 7 (1988) 3023–3029.
[245] S. Castorph, D. Riedel, L. Arleth, M. Sztucki, R. Jahn, M. Holt, T. Salditt, Structure parameters of synaptic vesicles quantified by small-angle X-ray scattering, Biophys. J. 98 (2010) 1200–1208.
[246] I. Augustin, C. Rosenmund, T.C. Südhof, N. Brose, Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles, Nature 400 (1999) 457–461.
[247] X. Lou, N. Korogod, N. Brose, R. Schneggenburger, Phorbol esters modulate spontaneous and Ca^{2+}-evoked transmitter release via acting on both Munc13 and protein kinase C, J. Neurosci. 28 (2008) 8257–8267.
[248] P. Chameau, Y. Van de Vrede, P. Fossier, G. Baux, Ryanodine-, IP_3- and NAADP-dependent calcium stores control acetylcholine release, Pflugers Arch. 443 (2001) 289–296.
[249] J.E. Richmond, W.S. Davis, E.M. Jorgensen, UNC-13 is required for synaptic vesicle fusion in *C. elegans*, Nat. Neurosci. 2 (1999) 959–964.
[250] H.T. McMahon, M.M. Kozlov, S. Martens, Membrane curvature in synaptic vesicle fusion and beyond, Cell 140 (2010) 601–605.
[251] M. Geppert, Y. Goda, R.E. Hammer, C. Li, T.W. Rosahl, C.F. Stevens, T.C. Südhof, Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse, Cell 79 (1994) 717–727.
[252] A.J. Groffen, S. Martens, R. Diez Arazola, L.N. Cornelisse, N. Lozovaya, A.P. de Jong, N.A. Goriounova, R.L. Habets, Y. Takai, J.G. Borst, N. Brose, H.T. McMahon, M. Verhage, Doc2b is a high-affinity Ca^{2+} sensor for spontaneous neurotransmitter release, Science 327 (2010) 1614–1618.
[253] Z.P. Pang, T. Bacaj, X. Yang, P. Zhou, W. Xu, T.C. Südhof, Doc2 supports spontaneous synaptic transmission by a Ca^{2+}-independent mechanism, Neuron 70 (2011) 244–251.
[254] J. Yao, J.D. Gaffaney, S.E. Kwon, E.R. Chapman, Doc2 is a Ca^{2+} sensor required for asynchronous neurotransmitter release, Cell 147 (2011) 666–677.
[255] F. Deák, O.H. Shin, J. Tang, P. Hanson, J. Ubach, R. Jahn, J. Rizo, E.T. Kavalali, T.C. Südhof, Rabphilin regulates SNARE-dependent repriming of synaptic vesicles for fusion, EMBO J. 25 (2006) 2856–2866.
[256] D.J. James, C. Khodthong, J.A. Kowalchyk, T.F. Martin, Phosphatidylinositol 4,5-bisphosphate regulation of SNARE function in membrane fusion mediated by CAPS, Adv. Enzyme Regul. 50 (2010) 62–70.
[257] M. Okamoto, T.C. Südhof, Mints, Munc18-interacting proteins in synaptic vesicle exocytosis, J. Biol. Chem. 272 (1997) 31459–31464.
[258] T. Biederer, T.C. Südhof, Mints as adaptors. Direct binding to neurexins and recruitment of Munc18, J. Biol. Chem. 275 (2000) 39803–39806.
[259] A. Maximov, T.C. Südhof, I. Bezprozvanny, Association of neuronal calcium channels with modular adaptor proteins, J. Biol. Chem. 274 (1999) 24453–24456.
[260] S.D. Fenster, W.J. Chung, R. Zhai, C. Cases-Langhoff, B. Voss, A.M. Garner, U. Kaempf, S. Kindler, E.D. Gundelfinger, C.C. Garner, Piccolo, a presynaptic zinc finger protein structurally related to bassoon, Neuron 25 (2000) 203–214.
[261] Y. Wang, M. Okamoto, F. Schmitz, K. Hofmann, T.C. Südhof, Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion, Nature 388 (1997) 593–598.
[262] P.J. Wen, S.L. Osborne, I.C. Morrow, R.G. Parton, J. Domin, F.A. Meunier, Ca2+−regulated pool of phosphatidylinositol-3-phosphate produced by phosphatidylinositol 3-kinase C2alpha on neurosecretory vesicles, Mol. Biol. Cell 19 (2008) 5593–5603.
[263] R.V. Stahelin, D. Karathanassis, K.S. Bruzik, M.D. Waterfield, J. Bravo, R.L. Williams, W. Cho, Structural and membrane binding analysis of the Phox homology domain of phosphoinositide 3-kinase-C2alpha, J. Biol. Chem. 281 (2006) 39396–39406.
[264] H. Liao, J. Ellena, L. Liu, G. Szabo, D. Cafiso, D. Castle, Secretory carrier membrane protein SCAMP2 and phosphatidylinositol 4,5-bisphosphate interactions in the regulation of dense core vesicle exocytosis, Biochemistry 46 (2007) 10909–10920.
[265] H. Zhang, L.C. Craciun, T. Mirshahi, T. Rohács, C.M. Lopes, T. Jin, D.E. Logothetis, PIP_2 activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents, Neuron 37 (2003) 963–975.
[266] B.C. Suh, B. Hille, PIP_2 is a necessary cofactor for ion channel function: how and why? Annu. Rev. Biophys. 37 (2008) 175–195.
[267] B.C. Suh, K. Leal, B. Hille, Modulation of high-voltage activated Ca^{2+} channels by membrane phosphatidylinositol 4,5-bisphosphate, Neuron 67 (2010) 224–238.
[268] P.A. Oude Weernick, M. López de Jesús, M. Schmidt, Phospholipase D signaling: orchestration by PIP_2 and small GTPases, Naunyn Schmiedebergs Arch. Pharmacol. 374 (2007) 399–411.
[269] M. Zeniou-Meyer, N. Zabari, U. Ashery, S. Chasserot-Golaz, A.M. Haeberlé, V. Demais, Y. Bailly, I. Gottfried, H. Nakanishi, A.M. Neiman, G. Du, M.A. Frohman, M.F. Bader, N. Vitale, Phospholipase D1 production of phosphatidic acid at the plasma membrane promotes exocytosis of large dense-core granules at a late stage, J. Biol. Chem. 282 (2007) 21746–21757.
[270] N. Brose, K. Hofmann, Y. Hata, T.C. Südhof, Mammalian homologues of *Caenorhabditis elegans* unc-13 gene define novel family of C2-domain proteins, J. Biol. Chem. 270 (1995) 25273–25280.
[271] A. Betz, U. Ashery, M. Rickmann, I. Augustin, E. Neher, T.C. Südhof, J. Rettig, N. Brose, Munc13-1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release, Neuron 21 (1998) 123–136.
[272] S. Chidambaram, J. Zimmermann, G.F. von Mollard, ENTH domain proteins are cargo adaptors for multiple SNARE proteins at the TGN endosome, J. Cell Sci. 121 (2008) 329–338.
[273] M.G. Ford, I.G. Mills, B.J. Peter, Y. Vallis, G.J. Praefcke, P.R. Evans, H.T. McMahon, Curvature of clathrin-coated pits driven by epsin, Nature 419 (2002) 361–366.
[274] J. Zheng, S.M. Cahill, M.A. Lemmon, D. Fushman, J. Schlessinger, D. Cowburn, Identification of the binding site for acidic phospholipids on the PH domain of dynamin: implications for stimulation of GTPase activity, J. Mol. Biol. 255 (1996) 14–21.
[275] K. Takei, O. Mundigl, L. Daniell, P. De Camilli, The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin, J. Cell Biol. 133 (1996) 1237–1250.
[276] M.G. Ford, B.M. Pearse, M.K. Higgins, Y. Vallis, D.J. Owen, A. Gibson, C.R. Hopkins, P.R. Evans, H.T. McMahon, Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes, Science 291 (2001) 1051–1055.
[277] B. Zhang, Y.H. Koh, R.B. Beckstead, V. Budnik, B. Ganetzky, H.J. Bellen, Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis, Neuron 21 (1998) 1465–1475.
[278] M. Metzler, V. Legendre-Guillemin, L. Gan, V. Chopra, A. Kwok, P.S. McPherson, M.R. Hayden, HIP1 functions in clathrin-mediated endocytosis through binding to clathrin and adaptor protein 2, J. Biol. Chem. 276 (2001) 39271–39276.
[279] C.Y. Chen, F.M. Brodsky, Huntingtin-interacting protein 1 (Hip1) and Hip1-related protein (Hip1R) bind the conserved sequence of clathrin light chains and thereby influence clathrin assembly *in vitro* and actin distribution *in vivo*, J. Biol. Chem. 280 (2005) 6109–6117.
[280] J.A. Parker, M. Metzler, J. Georgiou, M. Mage, J.C. Roder, A.M. Rose, M.R. Hayden, C. Néri, Huntingtin-interacting protein 1 influences worm and mouse presynaptic function and protects *Caenorhabditis elegans* neurons against mutant polyglutamine toxicity, J. Neurosci. 27 (2007) 11056–11064.
[281] S. McLaughlin, A. Aderem, The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions, Trends Biochem. Sci. 20 (1995) 272–276.
[282] Y. Aikawa, T.F. Martin, ARF6 regulates a plasma membrane pool of phosphatidylinositol 4,5-bisphosphate required for regulated exocytosis, J. Cell Biol. 162 (2003) 647–659.
[283] M. Krauss, M. Kinuta, M.R. Wenk, P. De Camilli, K. Takei, V. Haucke, ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type I gamma, J. Cell Biol. 162 (2003) 113–124.
[284] J.E. Hinshaw, Dynamin and its role in membrane fission, Annu. Rev. Cell Dev. Biol. 16 (2000) 483–519.