TEMPORAL AND SPATIAL COORDINATION OF EXOCYTOSIS AND ENDOCYTOSIS

Eckart D. Gundelfinger, Michael M. Kessels and Britta Qualmann

In secretory cells, exocytosis and compensatory endocytosis are tightly coupled membrane trafficking processes that control the surface area and composition of the plasma membrane. While exocytic and endocytic processes have been studied independently in great detail, at present there is much interest in understanding the mode of their coupling. This review discusses emerging insights into the coupling of these processes, both in the chemical synapses of neurons and in non-neuronal cells.

ENDOCRINE AND EXOCRINE CELLS
Endocrine cells secrete their products (hormones) into the blood or into surrounding tissues, whereas exocrine cells deliver their products (sweat, tears or mucus) into ducts or to the body surface.

CHEMICAL SYNAPSE
A highly specialized cell–cell junction that is designed for efficient communication between a nerve cell and its target cell, that is, another neuron, a muscle cell or a gland cell.

Leibniz Institute for Neurobiology, Department of Neurochemistry and Molecular Biology, Brenneckestr. 6, 39118 Magdeburg, Germany. e-mails: gundelfinger@ ifn-magdeburg.de; kessels@ifn-magdeburg.de; qualmann@ ifn-magdeburg.de

Specialized secretory cells, including neurons and non-neuronal cells (for example, ENDOCRINE AND EXOCRINE CELLS, mast cells and various egg cells), carry out stimulus-induced release of secretory molecules by regulated exocytosis. The membrane that is incorporated into the plasma membrane by exocytosis then needs to be retrieved by compensatory endocytosis. In particular, in the CHEMICAL SYNAPSES of neurons, which release large amounts of neurotransmitter through the exocytosis of SYNAPTIC VESICLES (SVs), these endocytic processes have to be very efficient to rapidly restore the functionality of the PRESYNAPSE. In addition, these endocytic pathways have to ensure the correct sorting of lipid and protein components to maintain the different composition of cell-surface and intracellular membranes. These interconnected processes of exocytosis and endocytosis in the presynapse are commonly referred to as the SV cycle (FIG. 1).

Compensatory endocytosis can occur through a clathrin-mediated mechanism. In addition, there seem to be alternative modes of regulated exocytosis/endocytosis, known as 'kiss-and-run', which do not include complete vesicle fusion and might not involve clathrin-mediated vesicle recycling[1] (FIG. 1).

As the molecular mechanisms of exocytosis and clathrin-mediated endocytosis have been reviewed in quite some detail[2–6], only the essentials are summarized here (BOXES 1,2). Instead, we focus on the fact that the

delivery of membrane 'packages' to the plasma membrane by exocytosis and their re-internalization by compensatory endocytosis must be tightly balanced in regulated secretory cells. For a long time, the mode of coupling of these two processes was inferred only indirectly. Recently, the application of new imaging techniques, detailed ultrastructural studies, and the discovery and characterization of molecular components that might link the exocytic and endocytic machinery have revealed new insights into the temporal and spatial coordination of exocytic and endocytic trafficking events in secretory cells. It is, however, still difficult to firmly link the findings that have been obtained with the various methods and technologies.

Kiss or collapse? A long-standing question
A controversial debate on how exocytic and endocytic processes are coupled arose in the early 1970s when two laboratories described the recycling of SVs at the frog neuromuscular junction[7–9]. One view was that SVs fuse completely with the presynaptic membrane during exocytosis ('collapse') and are then retrieved through a clathrin-dependent process that occurs lateral to the ACTIVE ZONE of neurotransmitter release[7,10] (FIG. 1a,b). The alternative view predicted a 'kiss-and-run' mode of neurotransmitter release, in which SVs release their contents through an open fusion pore and are then retrieved on the spot[8] (FIG. 1c). However,
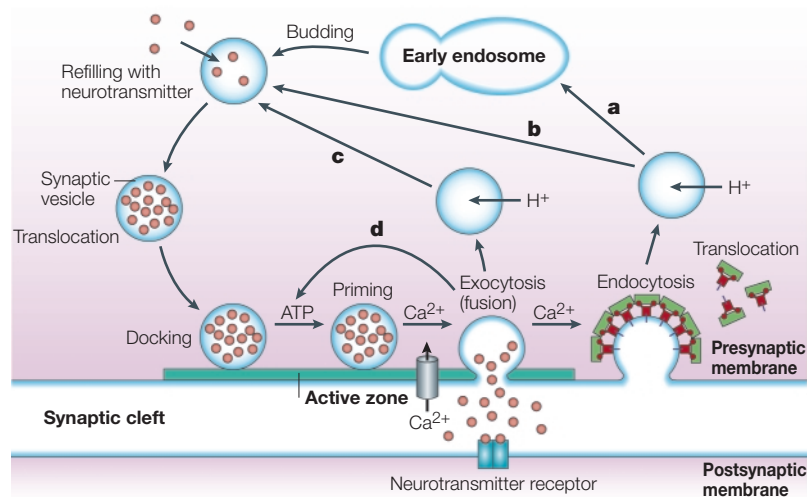
Figure 1 | **Exocytic and endocytic membrane trafficking events at the presynaptic membrane — the synaptic vesicle cycle.** Synaptic vesicles (SVs) are translocated to the active zone, tethered (docked) to the presynaptic membrane and primed (BOX 1). In response to Ca^{2+} influx, they undergo exocytosis and release neurotransmitters into the synaptic cleft, where these neurotransmitter molecules can bind to their receptors in the postsynaptic membrane. Various ways of retrieving the SV membrane have been reported. **a** | SVs could fuse and completely collapse into the presynaptic membrane and be retrieved by clathrin-mediated endocytosis (BOX 2). They could then be recycled through an endosomal compartment. **b** | Clathrin-mediated endocytosis could be followed by direct recycling, without passage through early endosomes. **c** | Alternatively, SVs might not fuse completely with the cell membrane, but might be rapidly retrieved and refilled after they have released neurotransmitters through a fusion pore. The molecular mechanism for this so-called 'kiss-and-run' mode of retrieval is essentially unknown. **d** | Finally, a 'kiss-and-stay' mode might be used, and this has been proposed for the rapid reuse of SVs[14]. In this case, SVs never leave the active-zone membrane, but are refilled instantly after closure of the fusion pore.

recent studies, indicate that there might even be vesicles that never leave the membrane of the active zone and that are almost instantly available for re-use[11–13], that is, these SVs must be refilled while they are attached to the plasma membrane. This mode, in which some of the vesicles only fuse transiently with the plasma membrane and seem to preserve their exocytotic machinery[13], would be an extreme shortcut of the kiss-and-run mechanism, and was named 'kiss-and-stay'[14] (FIG. 1d).

Some recent studies indicate that different modes of retrieval coexist in the PRESYNAPTIC BOUTONS of neurons and in non-neuronal secretory cells, and that the decision to pick a particular retrieval mode might be subject to cellular regulation. This might provide the necessary flexibility to a system that has to cope with a wide range of activities, from occasional stochastic release events to massive release that is induced by long and repetitive stimulation, or TONIC NEUROTRANSMITTER RELEASE at some highly specialized synapses.

Speed and timing of compensatory endocytosis

Non-neuronal cell models. Various electrophysiological monitoring methods and optical imaging techniques have been developed and applied to the study of the temporal coupling of exocytosis and endocytosis in secretory active cells (TABLES 1,2). Combining

electrophysiological recordings with the measurement of changes in membrane capacitance that are due to the fusion and retrieval of membrane packages and the detection of BIOGENIC AMINE release from mast or chromaffin cells by amperometry (TABLE 2) provides an excellent time resolution for these processes[15]. These cells therefore became prime models for studying the coupling of exocytic and endocytic processes[16–18]. Rapid endocytosis has time constants of a few hundred milliseconds to several seconds and is postulated to occur through the kiss-and-run mode, whereas full fusion might be followed by clathrin-mediated endocytosis, which takes minutes[17–19]. These pathways are subject to complex and differential regulation under conditions of physiological stimulation[19].

Neuronal cell models. Similar observations of the timing of compensatory endocytosis have been made at various chemical synapses (TABLE 1). Two different rates of endocytosis — a fast one (with time constants of ~1 s) and a slow one (with time constants of ≥10 s) — were reported from capacitance measurements at the RIBBON SYNAPSES of retinal bipolar cells[20–22] or inner-ear hair cells[23]. A careful capacitance analysis at the Calyx of Held (TABLE 1) revealed time constants of 56 ms after the fusion of a single SV, of 110 ms after a single ACTION POTENTIAL or low-frequency stimulation, and a gradual increase in the time constant to tens of seconds as the stimulus intensity was increased[24]. A similar lifetime of fusion pores has been proposed for small and LARGE DENSE-CORE VESICLES (LDCVs) in posterior pituitary neurons[25] (TABLE 1).

The optical imaging of fluorescent dyes (TABLE 2) has strongly promoted studies of membrane trafficking in the presynapse. Although the time resolution is not as good as with capacitance measurements, these techniques enable us to watch SVs at work[26,27] and to gain access to the many small conventional synapses, such as those that are formed between primary hippocampal neurons[11,12,28–30] (TABLE 1). FLUORESCENT STYRYL DYES, such as FM1-43 or FM2-10, can be taken up by SVs after their fusion with the cell membrane and subsequent endocytosis. By using excessive stimulation, a major proportion of SVs can be labelled and used to study SV recycling.

As different FM dyes have different membrane-dissociation kinetics, they can also be used to discriminate between kiss-and-run mechanisms, in which there is only a brief opening of a fusion pore (which releases the neurotransmitter[12] and FM2-10, but not FM1-43), and full fusion, which releases all components[11,28]. A series of elegant studies has confirmed that fast local cycling of SVs exists, with time-constants of only a few seconds[11,28,30]. Consistent with capacitance measurements at the Calyx of Held[24], the fraction of vesicles that participate in this fast cycling depends on the mode and intensity of stimulation[12,30].

Parallel endocytic pathways also coexist at the frog neuromuscular junction[31]. One relatively rapid pathway (taking <2 min) feeds retrieved vesicles directly into the rapidly recycling (or releasable) pool, while a second pathway (taking 10–20 min) feeds retrieved vesicles

Box 1 | The molecular mechanisms of exocytosis
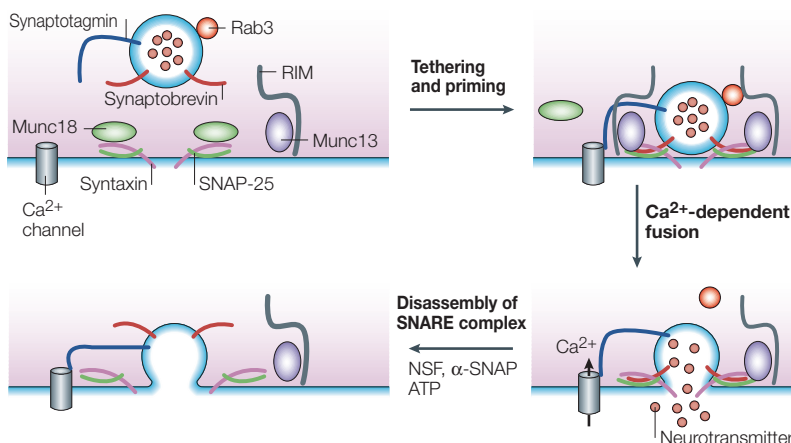
The fusion of membranes during exocytosis is considered as a two-step process — tethering (which is also known as docking) of an exocytic vesicle to the acceptor membrane and fusion of the lipid bilayers[2]. Tethering is mediated by members of the Rab family of small GTPases, which act in conjunction with various Rab effector proteins or tethering factors[134]. It is followed by the specific alignment of SNAREs (soluble *N*-ethyl-maleimide-sensitive fusion protein attachment protein receptors), which are anchored in the two membranes that are involved and mediate their fusion[2,3]. The assembly of the trans-SNARE complex is regulated by Munc18/Sec1 proteins, and disassembly is regulated by NSF (*N*-ethyl-maleimide-sensitive fusion protein) and α-SNAP (soluble NSF attachment protein).

Regulated exocytosis (for example, synaptic-vesicle or chromaffin-granule fusion) involves the SNAREs synaptobrevin/VAMP, SNAP-25 and syntaxin, and the small GTPase Rab3 (REFS 2,3). Tethering and fusion might be separated by a complex set of regulatory mechanisms (which are known as priming). Synaptic vesicles, for example, have to undergo a priming procedure, which involves Munc13 and Rab3-interacting molecules (RIMs), before they become fusion competent[10,103]. Fusion of primed vesicles occurs in response to Ca^{2+} influx into the cell through voltage-gated calcium channels. The Ca^{2+}- and phospholipid-binding protein synaptotagmin is thought to act as the Ca^{2+} sensor[88].

The figure shows the molecular mechanisms that are involved in regulated exocytosis (please note that the size of components is not drawn to scale and, to simplify the figure, not all of the components are depicted in each display).

BIOGENIC AMINES
A class of neurotransmitters that includes dopamine, adrenaline and noradrenaline, and 5-hydroxytryptamine (serotonin).

RIBBON SYNAPSE
A synapse that is specialized for the high-throughput release of transmitter; it is characterized by an elaborate, electron-dense presynaptic specialization (the synaptic ribbon), which mediates the efficient transport of synaptic vesicles to the active zone.

ACTION POTENTIAL
An electrical signal that travels along presynaptic neuronal extensions (axons) and elicits neurotransmitter release by depolarizing the presynaptic plasma membrane and triggering Ca^{2+} influx into presynaptic boutons.

through membrane invaginations and cisternae into the reserve (or resting) pool of SVs, from which vesicles can be only slowly recruited to the recycling pool (FIG. 2). However, it is unclear how these observations correlate with the much faster processes that are described above.

While it is tempting to assume that fast modes are kiss-and-run and/or kiss-and-stay mechanisms and might not involve the full fusion of SVs with the plasma membrane, a recent real-time imaging study provided evidence that fully fused vesicles might also be rapidly recycled in chemical synapses[32].

Spatial coordination of trafficking events

Structure of the site of exocytosis. The compartmentalization of membrane trafficking events and the establishment of a defined spatial organization for such events at the plasma membrane seem to be important concepts that cells use to ensure the order and efficiency of transport pathways. In neurons, this is achieved by organizing and concentrating special exocytic sites at active zones (FIG. 3a,b). Electron microscopy

(EM) studies showed that the active zone is characterized by a regular electron-dense structure that is adjacent to the presynaptic membrane[33] and might define the SV docking sites. Filamentous material originates from the presynaptic dense projection and extends deep into the synaptic cytosol (for a review, see REF. 34). It is probable that this specific array facilitates the intriguing efficiency of membrane transport processes in the synapse.

Live-imaging studies using evanescent-field video microscopy in goldfish retinal bipolar neurons have confirmed that exocytosis occurs repeatedly at the same preferred sites, which are presumably the active zones[26]. By contrast, in other regulated secretory cells, such as neuroendocrine cells, exocytosis seems to occur at random sites[35].

Sites of compensatory endocytosis. In neurons, the steady exocytosis of neurotransmitters must be accompanied by a compensatory endocytosis that retrieves both membrane and proteins to maintain cellular structure and function. There are similar needs in other regulated secretory cells. Interfering with compensatory endocytosis in lamprey axonal terminals (TABLE 1), by injecting antibodies against the endocytic machinery protein endophilin, had drastic effects on the structure and function of the presynapse. After persistent stimulation the SV pool was almost depleted and the plasma membrane was folded[36].

In general, the basic features and the molecular machinery of clathrin-dependent compensatory endocytosis seem to differ only slightly from those of constitutive or stimulated endocytosis (BOX 2). Endocytic components, however, are expressed at high levels in secretory cells and/or exist as specific isoforms, some of which exhibit distinct regulatory properties[4]. By contrast, the molecular machinery for kiss-and-run-type mechanisms is largely unknown. Similarly, the exact mechanisms by which exocytosis and endocytosis are coordinated at the synapse are still poorly understood.

The two fundamentally different endocytic pathways differ in their kinetics and their spatial organization. In kiss-and-run-type mechanisms, the sites of compensatory endocytosis should be identical to the sites of exocytosis (FIG. 1c). This has the advantage that components of the plasma membrane and of SVs do not mix and the geometric relationship of the proteins and lipids in SVs is maintained. As the process has been proposed to be clathrin-independent[17], it is difficult to identify the components of this pathway by EM or by immunocytochemistry, because vesicles that are endocytosed by the kiss-and-run or kiss-and-stay methods would not be distinguishable from SVs awaiting exocytosis. The spatial resolution of the optical imaging studies that are described above is not sufficient to detect the exact localization of retrieval sites relative to active zones.

Mechanistically, it remains unclear how spatial interference of the different processes that are operating in parallel is prevented, or how the required high-speed removal of empty vesicles from the active zone

Box 2 | The molecular mechanisms of clathrin-mediated endocytosis
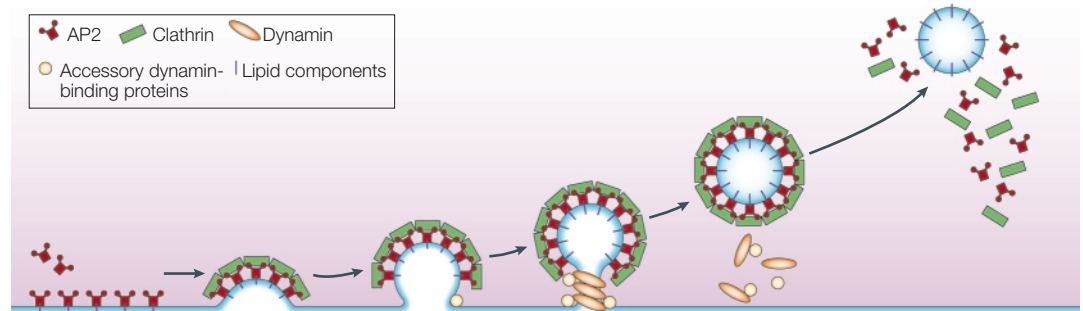
Generally, different forms of endocytosis, which can all occur through clathrin-mediated mechanisms, can be distinguished from each other. These processes are constitutive endocytosis, compensatory endocytosis and ligand-induced endocytosis (for example, of the epidermal growth factor receptor). The general sets of proteins that are required for these types of endocytosis are largely identical. The formation of clathrin-coated vesicles at the plasma membrane is driven by the assembly of clathrin triskelia into polyhedral lattices or cages (for a review, see REF. 135). Clathrin is recruited to the plasma membrane by the tetrameric adaptor protein (AP) complex AP2. The membrane association of AP2 is dependent on interactions with membrane phospholipids[55] (phosphoinositides), the integral membrane protein synaptotagmin[89] and specific sorting signals in the cytoplasmic tails of receptors and other cargo molecules (for a review, see REF. 5). Furthermore, clathrin-coat formation at the plasma membrane involves AP180, which binds to both clathrin and AP2 and has been proposed to regulate vesicle size. The subsequent formation of a deeply invaginated constricted coated pit, and its sequestration and detachment from the plasma membrane requires various additional proteins (for a review, see REF. 4). One is the large GTPase dynamin, which is targeted to the necks of endocytic coated pits where it participates in the fission reaction (for a review, see REF. 58). Clathrin-coated vesicles liberated from the donor membrane are then uncoated rapidly by an uncoating ATPase and moved into the cytosol, where they undergo further cellular sorting.

The figure shows the molecular mechanisms that are involved in clathrin-mediated endocytosis (please note that the size of components is not drawn to scale and that only the basic components are shown).

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is achieved. One solution would be that, under high frequency stimulation, exocytosis also occurs at sites other than the active zones in the presynapse. In goldfish retinal bipolar neurons, such 'non-active-zone' releases have been observed by evanescent-field microscopy, although most vesicles still docked and fused at predefined active zones[26].

For clathrin-mediated endocytosis, several studies over the past few years have reported a defined spatial segregation of exocytic and endocytic sites in nerve terminals. Components of the endocytic machinery, including dynamin and α-adaptin, are highly enriched in 'hot spots' of endocytosis in *Drosophila melanogaster* neuromuscular junctions[37–39], which indicates that the endocytic machinery is not freely diffusible, but is instead anchored in close proximity to exocytic zones (FIG. 3c). In line with this, EM studies of various types of synapses showed that invaginated coated pits occur at the edges of active zones of neurotransmitter release[7,36,40,41]. In snake neuromuscular junctions (TABLE 1), compensatory endocytosis-mediated dye uptake was observed at small punctate sites in the presynaptic membrane. The newly internalized coated vesicles and, to a lesser extent, coated pits appeared in clusters near, but somewhat outside, the active zones[42]. In unperturbed lamprey reticulospinal synapses (TABLE 1), coated pits can be detected within a radius of ≤0.5 μm from the edges of active zones. In

massively stimulated nerve terminals, however, in which endocytosis has been perturbed[40,41], coated pits in an arrested state were observed at an even greater distance from the edges of the active zones (FIG. 3b).

Endocytic sites are unlikely to be defined by cargo proteins. For example, in the nerve terminals of the temperature-sensitive *Drosophila* dynamin mutant SHIBIRE, which was kept at non-permissive temperature to block SV endocytosis, a redistribution of cargo molecules from SVs to the plasma membrane and their lateral diffusion did not affect the spatial organization of the recycling machinery[37,43]. Rather, components of the endocytic machinery itself might be spatial organizers.

The idea that endocytosis occurs at defined and specialized sites is supported by studies using green fluorescent protein (GFP)–clathrin and video microscopy in non-neuronal cells[44]. Similarly, activated G-protein-coupled receptor–β-arrestin complexes were endocytosed in pre-existing clathrin-coated pits instead of recruiting coat components to form new clathrin-coated pits[45,46].

Sea-urchin oocytes (TABLE 1) are a special case. In these cells, fertilization induces a massive exocytosis of CORTICAL GRANULES that is followed by compensatory endocytosis at exactly the same site[47]. The spatial cue for compensatory endocytosis is set directly by exocytosis, because, on vesicle fusion, Ca^{2+} channels are inserted

Table 1 | **Cellular systems used to study exocytosis and endocytosis**

System	Description	References
Non-neuronal model systems		
Sea urchin eggs	Egg cells that secrete material from cortical granules after fertilization. Exocytosis is followed by rapid compensatory endocytosis.	47,48
Panreatic β-cells	Endocrine cells that are embedded in the exocrine pancreas. They can be isolated by collagenase digestion of mouse pancreas. Inositol hexakisphosphate (InsP$_6$) increases Ca^{2+}-channel activity and acts as a trigger for insulin secretion from intracellular granulae. The system is under metabolic control.	67,68
Mammalian adrenal chromaffin cells	Cells of the adrenal medulla that release catecholamines from secretory vesicles (chromaffin granules) on stimulation.	13,16–19,50, 51,65,72,85, 99,119,122
Phaeochromocytoma (PC12) cells	Cells from an established neuroendocrine cell line that was originally derived from a chromaffin-cell tumour.	70, 98
Neuronal model systems		
Neuromuscular junction of *Drosophila melanogaster* larvae	Chemical synapse between the motorneuron and the larval body wall muscle, which releases glutamate that is stored in synaptic vesicles (SVs) from presynaptic boutons in response to incoming action potentials.	37–39,43, 86,90,93, 136
Neuromuscular junction of vertebrates (frog, snake)	Chemical synapse between the motoneuron and skeletal muscle, which releases acetylcholine from SVs in response to incoming action potentials.	7,8,31,42, 80
Lamprey reticulospinal synapse	Giant presynaptic terminal in the lamprey central nervous system, which is easily accessible to manipulation, for example, the microinjection of compounds in the vicinity of neurotransmitter-release sites.	36,40,41, 63,81
Nerve terminals of bipolar cells of the goldfish retina	Bulbous nerve terminals that can release large amounts of glutamate from SVs. Active zones are characterized by synaptic ribbons (highly specialized presynaptic cytomatrices) that extend into the cytoplasmic space and help to recruit and deliver SVs to their precise site of fusion.	20–22,26,120
Inner-ear hair cell afferent synapse in the murine cochlea	Ribbon synapse in mechanoreceptor cells of the inner ear that are designed to release large amounts of neurotransmitter to postsynaptic receptors of the auditory nerve.	23
Posterior pituitary nerve terminals	Nerve terminals in the posterior pituitary gland (neurohypophysis), which contain small SV-like vesicles and large dense-core vesicles that are filled with peptide homones (including oxytocin and vasopression).	25
Calyx of Held	Giant presynaptic nerve terminal in the auditory pathway of the mammalian brain. It contains several hundred release sites for glutamate from SVs.	24,113,114
Rodent primary hippocampal neurons	Neurons from the late embryonic or neonatal hippocampus that are dissociated and grown in culture for up to four weeks. They can differentiate and form synapses, which function as models for typical synapses that occur in myriads in the brain (the human brain is assumed to harbor 10^{14}–10^{15} conventional synaptic junctions). Most of these synapses use glutamate as the transmitter.	11,12,28–30, 74,121,123
Synaptosomes from rat cerebral cortex	Biochemical preparation of synaptic nerve terminals with adjacent postsynaptic specialization from the rat forebrain, which are still functional in neurotransmitter (glutamate) release.	124,125,127

into the plasma membrane. Endocytosis then seems to be triggered by activation of these Ca^{2+} channels, which might function as nucleating points for the endocytic machinery[48].

Morphological examinations of the spatial arrangement of different recycling pathways were carried out in nerve terminals of *Drosophila* retinula photoreceptor cells[49]. The authors of this study exploited the fact that, although rapid and slow compensatory endocytosis might differ mechanistically, dynamins seem to be involved in both processes[17,50]. Using the *shibire* mutant, they found two distinct SV recycling pathways. One emanated directly from the active zones and had a fast time course (~1 min), whereas the second, slower one (~30 min) occurred distal from the active zone (FIG. 2). The first pathway was blocked by exposure to high Mg^{2+} and low Ca^{2+} concentrations, while the second led to tubular invaginations when the dynamin-dependent pinch-off was blocked at non-permissive temperatures.

At present, it is unclear how the imaging data and the morphological data that are described above correlate, as they seem to operate at different timescales. Nonetheless,

all data support the view that there are at least two pathways of endocytic recycling and that they need to be coordinated and regulated individually, in relation to each other and in relation to the other membrane trafficking pathways in the small presynaptic compartment (FIG. 2). This adds significantly to the functional complexity of the system. In addition, the existence of dynamin-independent endocytic pathways, as reported for chromaffin cells[51], might increase the complexity further. The synapse seems to organize this wealth of trafficking pathways by compartmentalization, and by the use of a highly sophisticated molecular set-up that we are just beginning to understand.

Spatial design through membrane specialization

A defined spatial organization of membrane trafficking pathways in regulated secretory cells, and especially in the presynaptic compartment of neurons, requires distinct organizing elements, which must fulfil the following criteria: they have to act in or at the cell membrane; they must have the potential to interact with components of secretory vesicles; and they must display a regulated plasticity.

Table 2 | **Methods to monitor exocytosis and endocytosis**

Method	Description	References
Time-resolved capacitance measurement	Exocytic fusion of a vesicle with the plasma membrane (or opening of a fusion pore during exocytosis) results in an increased cell surface area and a corresponding increase in membrane capacitance and, vice versa, vesicle endocytosis results in a corresponding decrease. These changes in membrane capacitance can be measured with high time resolution in conventional whole-cell or perforated patch-clamp configurations.	For a review, see 15
Amperometry	This method can detect serotonin and catecholamines (that is, adrenaline, noradrenaline and dopamine). It relies on the current that is generated from the oxidation or reduction of the secreted product at the surface of a carbon fibre electrode, which is placed in the vicinity of the secreting cell. Amperometry is frequently combined with capacitance measurements.	For a review, see 15
Optical imaging of fluorescent dyes and fluorescently labelled proteins	This technique monitors fluorescent markers, for example, the styryl dyes FM1-43, FM4-64 or FM2-10, or proteins tagged with green fluorescent protein (GFP) or its derivatives using conventional or laser-scanning fluorescence microscopy.	For reviews, see 137,138
Evanescent wave or total internal reflection fluorescence microscopy	This method allows the real-time monitoring of fluorescent molecules in the evanescent field, that is, ~100 nm from the cell membrane into the cytoplasm.	For a review, see 27

Attractive candidates for such organizing elements are lipid components, the cortical cytoskeleton and specialized scaffolding molecules (FIG. 4). All three elements seem to be involved in coordinating the exocytic and endocytic machinery, and they seem to work together. Specialized lipids are part of both the plasma and vesicular membranes and their organizing role could be based on protein–lipid interactions, lipid rafts that create defined membrane domains and/or their many regulatory functions. The cortical cytoskeleton is a network of actin and spectrin fibres, which is attached to the plasma membrane. This network is dynamic, interacts with vesicles and responds to various stimuli including certain lipids. Finally, specialized scaffold molecules can create extended CYTOMATRIX structures, which might interact with and organize the molecular machinery for vesicle fusion and/or retrieval (FIG. 4).

Lipid domains. Although phosphatidylinositides are only a minor fraction of membrane phospholipids, they have emerged as key regulators of membrane trafficking and signalling events at the synapse[52]. Modulation of the synthesis, interconversion and degradation of membrane phosphatidylinositides is a powerful mechanism for the temporal and spatial regulation and coordination of signalling processes, membrane trafficking events and the actin cytoskeleton[52–54]. Several proteins that are involved in SV recycling contain phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$)-binding domains, such as PLECKSTRIN-HOMOLOGY (PH) domains[53].

In non-neuronal cells, localization studies using antibodies and GFP-labelled $PtdIns(4,5)P_2$-binding domains showed a non-uniform, raft-like distribution of $PtdIns(4,5)P_2$ on plasma membranes[54], which indicates that $PtdIns(4,5)P_2$-rich microdomains can establish discrete sites for vesicular trafficking (FIG. 4a). $PtdIns(4,5)P_2$ is involved in nearly all stages of clathrin-mediated endocytosis in neuronal and non-neuronal cells[52,53]. The initial stages of clathrin-coat formation depend on a $PtdIns(4,5)P_2$-mediated recruitment of the core clathrin coat and coat-associated proteins, which include AP2 (REF. 55), AP180 (REF. 56) and epsin[57]. The subsequent liberation of endocytic vesicles from the plasma membrane involves the large GTPase dynamin (BOX 2). The binding of $PtdIns(4,5)P_2$ to the PH domain of dynamin is crucial for its membrane recruitment and endocytic function[58]. Next, the local synthesis of phosphatidylinositol phosphates in membrane raft domains might be important in regulating actin polymerization-driven vesicle movement[59]. Finally, hydrolysis of $PtdIns(4,5)P_2$ by the polyphosphoinositide phosphatase synaptojanin[60] is required for the uncoating of newly formed vesicles. This has been shown by knockout studies in mice[61] and worms[62], and by microinjection studies using peptides and antibodies that interfere with the functions of synapojanin in lamprey synapses[63], which all resulted in an accumulation of clathrin-coated pits and/or vesicles in nerve terminals (FIG. 3b).

$PtdIns(4,5)P_2$ is also essential for exocytosis in several systems. In neuroendocrine cells, it is required for priming and, at later stages, presumably for the Ca^{2+}-triggered fusion of secretory granules with the plasma membrane[52,53]. During priming, phosphatidylinositol-transfer protein acts by binding to phosphatidylinositol-4-phosphate (PtdIns4P) and presenting it to a PtdIns4P-5-kinase, which generates $PtdIns(4,5)P_2$[64,65]. The Ca^{2+}-stimulated exocytosis of LDCVs from neuroendocrine cells is inhibited by the $PtdIns(4,5)P_2$-binding PH domain of phospholipase-Cγ, which is localized to the plasma membrane, but not to granules. This indicates that the plasma membrane pool of $PtdIns(4,5)P_2$ is important for exocytosis[66].

Reports indicate that, in addition to $PtdIns(4,5)P_2$, inositol hexakisphosphate ($InsP_6$) also might regulate both endocytosis and exocytosis. In pancreatic β-cells, $InsP_6$ is under metabolic control and promotes regulated insulin secretion[67]. Furthermore, $InsP_6$ is able to promote dynamin-dependent endocytosis. An increase in the amount of $InsP_6$ activates protein kinase C and inhibits synaptojanin in pancreatic β-cells, which leads to an increase in $PtdIns(4,5)P_2$ and indicates that $InsP_6$ might promote endocytosis through several different regulatory mechanisms[68].
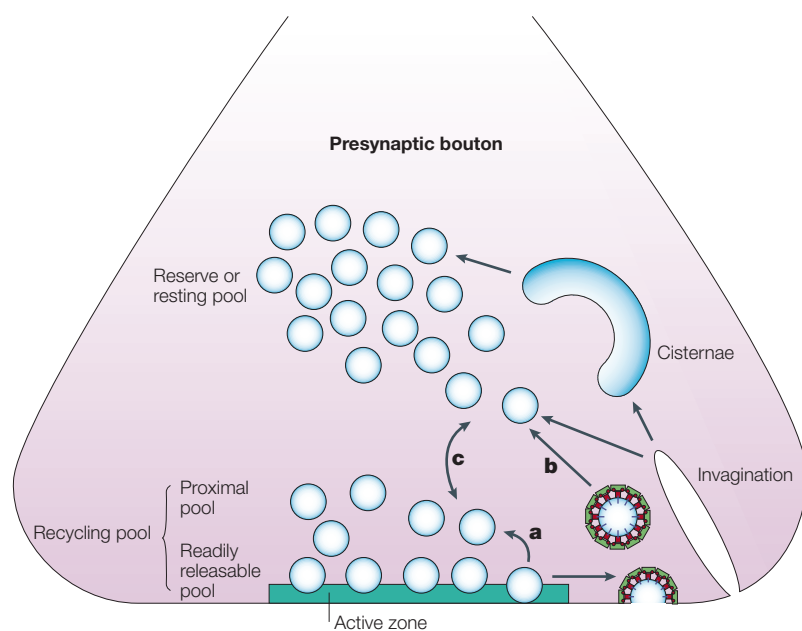
Figure 2 | Synaptic-vesicle pools in presynaptic boutons. According to their availability for neurotransmitter release and/or according to their localization in the nerve terminal, various pools of synaptic vesicles (SVs) can be distinguished[9,14,34]. Vesicles of the rapidly recycling pool participate actively in exocytosis under conditions of physiological stimulation, whereas the reserve or resting pool includes SVs that are activated only in response to strong synaptic stimulation. Vesicles of the recycling pool can be further divided into the readily releasable pool of SVs, which are docked and primed and can exocytose their content within milliseconds after stimulation, and the proximal pool[34,101], which can rapidly replace vesicles that have released their content. At a typical brain synapse, the rapidly recycling pool might consist of about 25 vesicles, of which 5–8 are readily releasable[14,29]. Different modes of coupling of exocytosis and endocytosis can feed into different SV pools. The 'kiss-and-run' mode is thought to feed into the recycling pool (**a**), whereas clathrin-mediated endocytosis feeds into the reserve pool (**b**). For example, at the neuromuscular junction of *Drosophila melanogaster* null-mutants for the *endophilin A* gene, in which pathway **b** is essentially blocked, about 15–20% of SVs are still cycling[136]. These authors propose that these vesicles reflect the recycling SV pool, which is maintained by an endophilin-independent kiss-and-run mode. Vesicles of the reserve or resting pool might replace aged cycling SVs or refill the recycling pool during extensive synaptic stimulation (**c**). A similar functional organization of excitatory vesicles has been described for adrenal chromaffin cells[16].

Finally, SV components to be re-internalized from the plasma membrane might be organized in lipid rafts[69]. This was indicated by studies showing that, in PC12 cells (TABLE 1), the formation of synaptic-like microvesicles was reduced by cholesterol depletion, without affecting horseradish peroxidase fluid-phase uptake[70].

The cortical cytoskeleton. Eukaryotic cells have a cortical actin–spectrin-based cytoskeleton that is underneath the plasma membrane. The extent and degree of crosslinking of the cytoskeleton vary among different cell types. For secretory vesicles, the actin–spectrin network forms a passive barrier that prevents their access to release sites. To allow selected vesicles to gain access to the plasma membrane, local disassembly of actin filaments needs to take place[71,72]. In regulated secretion, the stimulus-triggered increase of intracellular Ca^{2+} might regulate the organization of the cortical cytoskeleton through Ca^{2+}-dependent actin-binding

and -modulating proteins, for example, gelsolin and scinderin. Both of these proteins exhibit a Ca^{2+}-stimulated filamentous (F)-actin severing activity[72]. Scinderin associates with plasma-membrane phospholipids in a regulated manner, and $PtdIns(4,5)P_2$ competes with both the actin-binding and -severing activity of scinderin. This indicates that, on stimulation, scinderin might be released from the plasma membrane and activated to disassemble actin filaments in a coordinated way. As scinderin also promotes actin assembly in a Ca^{2+}-independent manner, it might also have a role in restoring the cortical actin network when the Ca^{2+} concentration decreases[72].

In nerve terminals, actin filaments were reported to disassemble and reassemble reversibly during SV recycling[73]. Neurotransmitter release from cultured hippocampal neurons was increased transiently by treatment with Latrunculin A (a drug that sequesters actin monomers), but not by treatment with Cytochalasin D (a drug that interferes with actin polymerization at the fast-growing end of actin filaments), which indicates the importance of shorter, more dynamic actin filaments in SV recycling[74].

In the endocytic process, however, actin is unlikely to only have an inhibitory role. The generation of force by actin polymerization might support different stages of endocytic processes, including vesicle fission, detachment from the plasma membrane and the movement of newly formed endocytic vesicles into the cytoplasm[6,75] (BOX 2). Recently, live-imaging studies showed that actin associates with endocytic structures when they detach from the plasma membrane[76].

Another function of the cortical cytoskeleton might be the spatial organization of membrane trafficking events, either by passively encasing exocytic and/or endocytic machineries or by actively anchoring and connecting the machineries through physical interactions with some of their components[6,75]. To fulfil such an organizational role, the cytoskeleton needs to provide defined spatial clues, and therefore must not be organized in a homogenous manner. Cortical cytoskeletal structures might therefore be either organized in a discontinuous network, leaving 'holes' for membrane trafficking events, or provide specific attachments sites for multifunctional scaffolding proteins — such as syndapin[77], Huntingtin-interacting protein 1-related (HIP1R)[78], intersectin[39] and Abp1 (actin-binding protein 1)[79] — which link the cytoskeleton to membrane trafficking machineries (FIG. 4b). These molecular links might recognize specific states of the cytoskeleton, for example, different degrees of polymerization, bundling and/or crosslinking. In line with a spatial organizing role for the actin cytoskeleton, the very limited lateral mobility of clathrin-coated pits increased on Latrunculin B treatment[44]. This indicates the removal of some kind of actin cytoskeletal constraint.

A high degree of spatial organization of the machineries for stimulated exocytosis and compensatory endocytosis at the plasma membrane has been observed at presynaptic boutons (FIG. 3). This might ensure the high speed and efficiency of membrane trafficking processes

in SV recycling. At synaptic sites in the frog neuromuscular junction, F-actin and β-fodrin appear concentrated in a ladder-like pattern at the non-release domains[80]. These domains probably correspond to endocytic hot spots that contain high concentrations of endocytic coat and accessory proteins, as has been observed at the *Drosophila* neuromuscular junction[43] (FIG. 3c). Recent EM studies of lamprey reticulospinal axons showed stimulus-dependent reorganization of actin filaments, which

Figure 3 | **Spatial organization of the sites of exocytosis and endocytosis in nerve terminals.** Parts **a** and **b** show electron micrographs from lamprey reticulospinal synapses. **a** | An unperturbed synapse that has a large cluster of synaptic vesicles (SVs; marked by a white asterisk) in the active zone. The bar represents 250 nm. Note that under the normal conditions that are shown here, no clathrin-coated endocytic intermediates are preserved during specimen preparation and fixation. Reproduced with permission from REF. 63 © (2000) Elsevier Science. **b** | Microinjection of a peptide that comprises the endophilin-binding site of synaptojanin blocks clathrin-mediated endocytosis at different steps and therefore synaptic transmission after high-frequency stimulation. Note that, in such a specimen, the SV pool is almost depleted (black asterisk) and that arrested clathrin-coated intermediates are visible in the presynapse (arrows). The bar represents 250 nm. Image kindly provided by O. Shupliakov, L. Brodin and H. Gad, Karolinska Institute, Stockholm, Sweden. **c** | The neuromuscular junctions of *Drosophila melanogaster* larvae have a functional organization in which endocytic 'hot spots' (marked with anti-DAP160/intersectin staining; red) are closely neighboured, and often surrounded, by sites of exocytosis (marked with antibodies against the glutamate receptor subunit GluRB staining; green). Image kindly provided by J. Roos, Neurogenetics Inc., La Jolla, California, USA, and reproduced with permission from REF. 75 © (2000) Rockefeller University Press.

seemed to be associated with clathrin-coated vesicles that were forming lateral to the SV-enriched active zones[81]. These observations are consistent with a role for the cortical cytoskeleton in either compensatory endocytosis or in restricting SVs to release domains. In presynaptic active zones, two chief components of the presynaptic dense projection, which is thought to be important for the localization and fusion of SVs, are actin and spectrin[82]. Several interactions between cytoskeletal components and molecules that are involved in SV exocytosis have been reported. The SV-priming protein Munc13 interacts with a brain-specific β-spectrin isoform[83], and syntaxin interacts with α-fodrin[84], which has been implicated in stimulated secretion in chromaffin cells[85]. Consistent with this, in *Drosophila*, α- or β-spectrin null mutants displayed severely disrupted neurotransmission at neuromuscular junctions without altered synapse morphology[86].

Immunofluorescence studies in hippocampal neurons indicate the interesting possibility that the sites of endocytosis and exocytosis in presynaptic boutons might contain different actin pools that correspond to distinct functional stages, and therefore, perhaps, to different functions. The presynaptic actin pool that colocalizes with the active-zone protein Bassoon is not labelled efficiently with phalloidin[74], a reagent that binds only F-actin. This indicates either that actin-binding proteins, which might be enriched locally, compete with phalloidin at active zones or that the actin pool at exocytic sites is kept primarily in a monomeric state.

F-actin might be important for the organization of membrane trafficking processes primarily in the presynaptic terminals of developing, immature neurons. In hippocampal neurons, F-actin depolymerization by Latrunculin A treatment during the first week of cell culture resulted in drastic changes exclusively in synaptic ultrastructure and in a complete loss of SYNAPTOPHYSIN-labelled vesicle clusters and synaptic-recycling activity. By contrast, after 18 days, SV recycling and synaptophysin clusters were largely resistant to this treatment[87]. This decreased sensitivity to actin-filament depolymerization correlated with an increased clustering and actin filament-independent retention of the presynaptic cytomatrix protein Bassoon. These results indicate that, in mature synapses, both the maintenance of ultrastructure and the localization of many synaptic proteins have become independent of F-actin, and that these functions might have been taken over by scaffolding molecules of the 'cytomatrix assembled at the active zone' (CAZ), which is discussed in more detail below.

Specialized scaffolding structures. The spatial restriction of SV exocytosis to active zones and the organization, maintenance and replenishment of different SV pools near these sites, as well as the speed and efficiency of synaptic transmission, could be achieved by dynamic macromolecular complexes that are based on different protein–protein and protein–lipid interactions (FIG. 4c).

a Lipid components   **b** The cortical cytoskeleton   **c** Specialized scaffolding proteins

PtdIns(4,5)P$_2$
PtdIns(4,5)P$_2$-binding proteins
InsP$_6$
InsP$_6$-regulated proteins
Lipid rafts

Actin
Spectrin/Fodrin
HIP1R
Abp1
Syndapins

Bassoon
Piccolo
RIMs
Munc13s
CAST

Figure 4 | **Elements with an ability to compartmentalize the plasma membrane and thereby organize the machineries for membrane trafficking processes. a** | Lipid components (blue) within the plasma membrane might provide spatial cues for vesicle (yellow) docking or endocytosis. **b** | The cortical actin/spectrin cytoskeleton (brown) that underlies the plasma membrane compartmentalizes the continuum of the membrane and can function as a framework for proteins (different shades of red) that link it to membrane trafficking processes. **c** | Specialized cross-connected scaffolding proteins (different shades of purple) that are associated with the plasma membrane can mark certain sites and organize locally both the exocytosis and/or endocytosis machinery and different vesicle pools, for example, at the active zone for neurotransmitter release. Under each part of the figure, key examples of each type of organizing element are shown. Abp1, actin-binding protein 1; CAST, CAZ-associated structural protein; HIP1R, Huntingtin-interacting protein 1-related; InsP$_6$, inositol hexakisphosphate; PtdIns(4,5)P$_2$, phosphatidylinositol-4,5-bisphosphate; RIMs, Rab3-interacting molecules.

C2 DOMAIN
(conserved region 2 of protein kinases C). C2 domains are ~100 amino-acids long and bind phospholipids and Ca^{2+} interdependently. They are present in many proteins that are involved in Ca^{2+} signalling.

STONED PROTEINS
Proteins encoded by the *stoned* locus of *Drosophila melanogaster* and their vertebrate homologues.

SRC-HOMOLOGY-3 DOMAIN
(SH3). A protein module of ~50 amino acids that can interact with the proline-rich motifs of proteinaceous binding partners.

SNARES
(soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors). A family of membrane-tethered coiled-coil proteins that regulate membrane fusion events.

Biochemical and genetic studies have implicated synaptotagmins in the modulation of both exocytic and endocytic events. Synaptotagmin I is a member of the large synaptotagmin protein family and is thought to be an important Ca^{2+} sensor for exocytosis in the synapse (for a review, see REF. 88). The potential involvement of synaptotagmin I in endocytosis could be mediated through interactions with AP2 (REF. 89) and with proteins that are encoded by the *stoned* locus of *Drosophila*[90] and their mammalian homologues (stoned/stonin). Indeed, overexpression of the transmembrane domain of synaptotagmin[91], or of a peptide that interferes with the interaction of AP2 with the second C2 DOMAIN (C2B) of synaptotagmin[92], resulted in dominant-negative effects on clathrin-dependent receptor-mediated endocytosis in non-neuronal cells. In addition, STONED PROTEINS localize to endocytic hot spots at *Drosophila* neuromuscular junctions, and might participate in endocytosis, as indicated by dye-uptake studies in fly mutants[93]. Interestingly, transgenic overexpression of synaptotagmin I rescues both the *stoned* embryonic lethality and the endocytic defect at the synapses.

Mutational analyses in *Drosophila* have revealed two independent functions of the synaptotagmin C2B domain in SV recycling. Deleting the C2B domain disrupted synaptotagmin–AP2 interactions and markedly reduced the number of SVs at mutant terminals during stimulation. This indicates that there is a selective impairment of SV endocytosis. By contrast, a point mutation in the C2B domain reduced both the Ca^{2+}-dependent dimerization of synaptotagmin and the ability of docked vesicles to fuse with the plasma membrane[94].

Similarly, intersectins — a family of large multidomain proteins — can associate with proteins of the endocytic and exocytic machinery in extracts from neuronal or non-neuronal cells. Their carboxy-terminal SRC-HOMOLOGY-3 (SH3) DOMAINS mediate interactions with dynamin and synaptojanin[39,95]. In line with this, an excess of the SH3 domains of intersectins or of the full-length proteins caused dominant-negative effects on endocytosis[95,96]. A potential role of intersectins in endocytosis is also indicated by the localization of the *Drosophila* intersectin homologue DAP160 in endocytic hot spots at neuromuscular junctions[39].

In addition, complexes of intersectin with the SNARE (soluble *N*-ethyl-maleimide-sensitive fusion protein attachment protein receptor) protein SNAP-25 (BOX 1) were detected by co-immunoprecipitation[97]. It has yet to be determined whether intersectins, SNAP-25 and endocytic proteins are present in one complex or form different intersectin-containing subcomplexes, and whether intersectins associate with free SNAP-25 or with SNAP-25 that is integrated into the exocytosis core complex.

A binding partner of the SV-associated GTPase Rab3a — rabphilin — also has links to both endocytosis and exocytosis. Overexpression of the Rab3a-binding region of rabphilin inhibits clathrin-mediated endocytosis in PC12 and HeLa cells[98]. In secretory cells, overexpression of full-length rabphilin stimulated exocytosis, whereas rabphilin C2-domain deletion mutants had a dominant-negative effect on secretion[99]. However, neither synaptic transmission nor plasticity were affected in *rabphilin*-deficient mice[100], which indicates that

there is only a minor or redundant role for rabphilin in SV recycling.

Several proteins, including Munc13s, Rab3-interacting molecules (RIMs), CAST (CAZ-associated structural protein), Bassoon and Piccolo/Aczonin, are localized specifically to the electron-dense CAZ[101,102]. This localization indicates that these proteins might have functions in SV exocytosis and in the spatial organization of neurotransmitter release[34,101,103]. For RIM, CAST and Munc13, whole networks of interacting proteins have been described that indicate an involvement of these proteins in different steps of the exocytic process[102–105]. Genetic knockout studies in several organisms have confirmed the importance of RIM and Munc13 in SV exocytosis, as disruption of these genes reduced both evoked and spontaneous neurotransmitter release[103,105,106]. The loss of either of these proteins did not, however, affect the density and size of synapses, the morphology of active zones, the density or the number of morphologically docked SVs[105,106], which either questions whether there is a general role for these proteins as spatial organizers at active zones or points to functional redundancies.

At present, the function of the two related proteins Bassoon[107] and Piccolo[108,109] is unknown. Their large size (420 kDa and 530 kDa, respectively) and multidomain structure would allow them to reach from the site of SV release to distant vesicle pools, and to form numerous interactions with other proteins. This indicates that they might organize the CAZ by forming large scaffolding complexes. It is thought that the CAZ function includes close cooperation with the cortical cytoskeleton. This might provide the basis for dynamic and rapid reorganization of presynaptic nerve terminals. Direct interconnections of the CAZ proteins with cytoskeletal elements have been identified; for example, Munc13 associates with a brain-specific isoform of β-spectrin[83] and Piccolo/Aczonin interacts with profilin[108], a small G-actin-binding protein. Furthermore, Piccolo also interacts with Abp1 (S. D. Fenster, M.M.K., B.Q. and C. C. Garner, unpublished observations), an F-actin binding protein that is thought to be involved in receptor-mediated endocytosis through an interaction with the GTPase dynamin[79].

Ca²⁺ — a key regulator of coupling?

Depolarization of the presynaptic nerve terminal by an action potential causes the influx of extracellular Ca²⁺ through voltage-gated calcium channels. The local increase of intracellular Ca²⁺ to 10–100 μM in a limited microdomain (~tens of nanometers) around clustered channel pores acts as a trigger for the exocytosis of neurotransmitters[110]. Molecules that function as Ca²⁺ sensors in exocytosis are therefore expected to be localized close to Ca²⁺ channels and to exhibit fast, but relatively low affinity Ca²⁺ binding.

The SV transmembrane protein synaptotagmin is the prime candidate to act as a Ca²⁺ sensor for exocytosis in the synapse. Ca²⁺-binding to the C2 domains of synaptotagmin regulates several of its lipid and protein interactions. Synaptotagmin can form Ca²⁺-dependent

complexes with isolated SNARE proteins and assembled SNARE complexes[88,110]. The C2A domain of synaptotagmin shows Ca²⁺-dependent rapid phosphoinositide binding with an EC_{50} that corresponds interestingly to the Ca²⁺ levels that are required to trigger SV exocytosis[111–114]. In line with these data, mutating the C2A domain reduced both neurotransmitter release and the ability of synaptotagmin I to bind to membrane lipids[115]. The C2-domain-containing proteins double C2 protein (DOC2), Munc13, rabphilin, RIM and Piccolo might be further Ca²⁺ sensors that are present in the synapse[34,101,116].

Moreover, the Ca²⁺-activated protein for secretion (CAPS) is essential for the fusion of LDCVs with the plasma membrane, as shown in PC12 cells[117] and synaptic terminals[118], and, at least in *Drosophila*, for the regulated release of glutamate at the neuromuscular junction[118]. For the secretion of dense-core vesicles, however, some of the Ca²⁺ effects on exocytosis might be mediated by the actin cytoskeleton.

Reports on the sensitivity of compensatory endocytosis to Ca²⁺ have been contradictory, and have indicated that endocytosis might be either activated or inhibited by manipulations that elevate internal Ca²⁺ or that they might be Ca²⁺-independent[16,119–123]. These differences might be due to different cell types and/or preparations, and perhaps reflect the different endocytic pathways that are used. Interestingly, altering the Ca²⁺ concentration in both chromaffin cells[18] and retinal bipolar cells[22] shifted the mode of recycling. In chromaffin cells, raising the extracellular Ca²⁺ concentration shifted the preferred mechanism of recycling to kiss-and-run, and the authors of this study presented a model in which Ca²⁺ regulates the opening time of the fusion pore. In this model, reclosure is rare at low Ca²⁺ concentrations, so the vesicle membrane might collapse completely into the plasma membrane, whereas high Ca²⁺ concentrations, which are present during periods of high activity, have been suggested to be the trigger for faster recycling modes, such as kiss-and-run[18].

In the presynaptic boutons of the lamprey giant reticulospinal axon, persistent stimulation can cause the massive incorporation of SV membranes into the plasma membrane. Retrieval of SV membranes can be blocked by removing external Ca²⁺, but can be restored by adding low external Ca²⁺, even in the absence of action potentials[40]. In several studies of compensatory endocytosis in chromaffin cells[19,122], and of styryl-dye uptake in either synaptosomes[124] or hippocampal synapses[28], endocytic activity was enhanced by Ca²⁺ influx and reduced by inhibitors of the Ca²⁺-sensitive phosphatase calcineurin. It is thought that calcineurin might be the Ca²⁺ sensor for endocytosis: elevation of internal Ca²⁺ activates calcineurin, which then causes the dephosphorylation of various endocytic proteins including dynamin, synaptojanin and amphiphysin[60,125,126]. Further results indicate that a phosphorylation cycle is necessary for SV recycling[127]. This phosphorylation cycle, in turn, regulates protein–protein and protein–lipid interactions; the latter are important for both the recruitment of endocytic

EC_{50}
The EC_{50} defines the ligand (for example, Ca²⁺) concentration that elicits 50% of the maximum possible activity of a biochemical reaction (for example, exocytic activity).

proteins to the presynaptic plasma membrane during endocytosis and their subsequent release. In resting nerve terminals, proteins of the endocytic machinery are largely in their phosphorylated form. Phosphorylated adaptins and dynamin 1 are localized exclusively to the cytosol in resting nerve terminals[128,129], whereas the dephosphorylation of coat proteins and accessory endocytic proteins correlates with their assembly into large protein complexes that are destined to catalyse the endocytic reaction[128,130].

The different Ca^{2+} sensitivities of the proposed Ca^{2+} sensors for endocytosis and exocytosis could also have consequences for the spatial organization of these membrane trafficking processes at the presynapse. Synaptotagmin has a K_d for Ca^{2+} that is in the micromolar range and would therefore require high Ca^{2+} concentrations, which are proposed to be present in the microdomains around activated Ca^{2+} channels. By contrast, calcineurin, which has a K_d in the hundreds-of-nanomolar range, might also be activated by cytoplasmic Ca^{2+} concentrations further away from these channels.

Conclusion and perspectives

We have only just begun to understand how exocytosis and endocytosis are spatially and temporally organized, and it is therefore still not understood how they are functionally coupled in all the various cellular systems. Our first glimpse of these processes has revealed that there is an unexpected variability in the mode of coupling and that there are numerous ways of organizing and regulating the different pathways. The fact that

many studies have used single specialized techniques and experimental systems makes it very difficult to correlate the results that have been obtained by different studies. Moreover, as many investigators have used unusually strong stimuli or non-physiological conditions, it is also clear that we are still far away from understanding the physiological situations — temperature, for example, affects SV cycling markedly[131].

Chemical synapses are enormously plastic, which is crucial for all major brain functions including learning and memory. The sophisticated mechanisms of coordinating exocytic and endocytic processes might contribute much more to synaptic plasticity than was previously thought — for example, mice that can release neurotransmitter, but not modulate this release, are not viable[132]. As the cycling of AMPA-TYPE GLUTAMATE RECEPTORS contributes significantly to synaptic plasticity[133], it seems probable that there is a similar complex interplay of exocytosis and endocytosis on the postsynaptic side, for example, to regulate the surface availability of neurotransmitter receptors.

Future studies must be aimed at uncovering the general principles of exocytic and endocytic coordination in secretory cells and to distinguish them from the more specific adaptations of individual secretory systems. This, however, will require a detailed understanding of the molecular mechanisms of all modes of exocytosis and endocytosis and their cross-connections, as well as their links to the structural components of different secretory cells — this is a challenge that will require strong interdisciplinary efforts.

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