Protein scaffolds in the coupling of synaptic exocytosis and endocytosis

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Abstract | Mechanisms that ensure robust long-term performance of synaptic transmission over a wide range of activity are crucial for the integrity of neuronal networks, for processing sensory information and for the ability to learn and store memories. Recent experiments have revealed that such robust performance requires a tight coupling between exocytic vesicle fusion at defined release sites and endocytic retrieval of synaptic vesicle membranes. Distinct presynaptic scaffolding proteins are essential for fulfilling this requirement, providing either ultrastructural coordination or acting as signalling hubs.

Active zone
(Often abbreviated to AZ.) An area in the presynaptic compartment that is specialized for rapid exocytosis and contains multidomain proteins acting as scaffolds in the organization of release sites.

Periactive zone
An array of endocytic proteins that surround the active zone and into which synaptic vesicle membranes are recycled following exocytosis.

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doi:10.1038/nrn2948
Published online
9 February 2011

Communication between nerve cells largely occurs at chemical synapses — specialized sites of cell–cell contact where electrical signals trigger the exocytic release of neurotransmitter, which in turn activates postsynaptic receptor channels. The efficacy with which such chemical signals are transmitted is crucial for the functioning of the nervous system. Modulation of neurotransmission enables nerve cells to respond to a vast range of stimulus patterns. Synaptic activity can also be tremendously diverse; from the fast synapses of the hippocampus, to slow neuropeptide-containing synapses. Indeed, some signalling pathways are active in the absence of stimulation, such as those involved in the processing of sensory information, which transmit tonically at high rates of up to 100 Hz or more[1,2].

Intriguing questions arise from these facts, including how high rates of neurotransmission can be maintained over long periods of time, and what limits the ability of a given synapse to release neurotransmitter during sustained periods of activity. Recent data indicate that in many synapses, exocytosis of neurotransmitter is coupled to endocytosis, and that synapses have evolved a specialized apparatus of scaffolding proteins to comply with these demands. Such scaffolds aid the temporal and spatial coupling of exocytosis and endocytosis, and are crucial for maintaining rapid neurotransmission during sustained activity[3].

Exocytosis of neurotransmitter is triggered by stimulus-induced calcium influx into the nerve terminal[4,5] and the subsequent fusion of synaptic vesicles with the presynaptic plasma membrane at specialized regions called active zones (BOX 1). Exocytosed synaptic vesicle membrane proteins and lipids in turn are recycled at the endocytic or periactive zone (BOX 1) that surrounds the release site, to restore functional synaptic vesicle pools for reuse and to ensure long-term functionality of the synapse[6–8] (FIG. 1). Depending on the type of synapse and the stimulation frequency, several modes of endocytosis with different time constants — for example, fast and slow endocytosis — seem to operate[7,9,10]. Clathrin-mediated endocytosis arguably represents the main pathway of synaptic vesicle endocytosis[6,8,11], although other modes — for example, fast kiss-and-run exocytosis and endocytosis — could operate in parallel.

Although the machineries for exocytic membrane fusion[12,13] and for endocytic retrieval of synaptic vesicle membranes have been studied separately in some detail[6,14], comparatively little is known about the coupling between exocytosis and endocytosis. Here we synthesize recent data from physiological, morphological and biochemical studies in several model systems into hypothetical models for scaffold-based mechanisms underlying exocytic–endocytic coupling.

The synaptic vesicle cycle

Synaptic vesicle pools. Some defining features of chemical synapses are the presence of one or several clusters of synaptic vesicles in the presynaptic nerve terminal, and ultrastructural specializations at the pre- and postsynaptic membranes[8]. Synaptic vesicles participate in a local cycle of regulated exocytosis and endocytosis that has drawn the attention of neuroscientists for more than three decades (FIG. 1). Synaptic vesicles are the morphological counterpart of the quantal release of neurotransmitter that was postulated by Katz more than 60 years ago[15].

Proteomic and lipidomic analysis[16] has revealed that an average 42-nm sized synaptic vesicle contains about

Box 1 | **Presynaptic organization — exocytic and endocytic zones**

Functional chemical synapses depend on the interplay of specialized membrane sites or subdomains. At the presynaptic side, the active zone provides the platform for rapid SNARE (soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein (SNAP) receptor) protein-driven fusion of neurotransmitter-filled synaptic vesicles after Ca^{2+} influx[109,110]. The active zone membrane is decorated by proteinaceous scaffolds characterized by a set of intracellular specialized proteins termed the cytomatrix of the active zone (CAZ). Accumulating evidence suggests that the CAZ comprises a small set of conserved large multidomain proteins that provide building blocks for a two-dimensional electron-dense matrix decorating the active zone of both vertebrate and invertebrate synapses[110,111]. These scaffolds might confer long-term stability (tenacity) to individual synaptic sites[112]. Moreover, CAZ components might be responsible for the close temporal coupling between the influx of Ca^{2+} through voltage-gated Ca^{2+} channels (localized and enriched at the active zone membrane) and the fusion of synaptic vesicles, perhaps by maintaining spatial proximity between Ca^{2+} channels and readily releasable synaptic vesicles[5,113].

The periactive zone is a specialized membrane domain surrounding the active zone and is characterized by an enrichment of endocytic proteins such as intersectin, epidermal growth factor receptor substrate 15 (EPS15), dynamin and endophilin. Within the periactive zone, exocytosed synaptic vesicle proteins are translocated and then recaptured by endocytic sorting adaptors, including stonin 2 (also known as stoned B) and perhaps AP180. It is likely that these adaptor proteins in turn are spatiotemporally organized by large multidomain scaffolds such as intersectin and EPS15 (REFS 62,72). The periactive zone also contains cytoskeletal elements, in particular actin and perhaps septin filaments. How precisely scaffolds of the active and periactive zones are functionally and structurally coupled remains an important subject for future studies.

1500–2000 neurotransmitter molecules. Most synaptic vesicle proteins are present in surprisingly low copy numbers per vesicle — in some cases, such as the synaptic vesicle 2-related protein (SV2) or the V-ATPase, just one or two are present. Functionally, at least three different pools of synaptic vesicles have been defined[17]. First, the readily-releasable pool (RRP) of synaptic vesicles is available immediately upon stimulation but depletes rapidly during high-frequency stimulation of the neuron (BOX 1). Morphologically and biochemically, the RRP may correspond to vesicles that are docked to the presynaptic active zone (BOX 1; see below). These vesicles are primed for release in that they have assembled a release apparatus that only needs a Ca^{2+} trigger to initiate exocytosis. In most synapses, this pool makes up only a few percent of the total number of synaptic vesicles. Second, the recycling pool of synaptic vesicles maintains release during moderate stimulation of the neuron and displays a much more scattered distribution within the nerve terminal[17]. Third, the reserve pool, which makes up the majority of vesicles within the synaptic vesicle cluster, is thought to constitute a depot of vesicles, the mobilization of which can be controlled by cell division protein kinase 5 (CDK5)[18]. The extent to which reserve pool vesicles participate in neurotransmitter release during sustained periods of high-level activity remains a matter of debate[19–21]. Vesicle numbers and pool sizes are quite similar for different glutamatergic synapses when normalized to the number of active zones in the synapse — regardless of whether a synapse has one to two active zones (for example, hippocampal synapses) or 500–600 active zones (for example, the calyx of Held)[9].

Release site
Docking sites for synaptic vesicles within active zones that can be empty and accessible for a vesicle, occupied and ready for fusion, or empty and inaccessible.

Cytoplasmic matrix of the active zone
(Often abbreviated to CAZ.) An electron-dense largely detergent-resistant matrix comprising multidomain proteins that may form release sites for exocytosis.

SNARE proteins and exocytosis: the nuts and bolts of membrane fusion. Neurotransmitter release is initiated by the depolarization-induced opening of presynaptic Ca^{2+} channels that are concentrated at defined release sites within the active zone[3,8,22]. The steep rise of intracellular Ca^{2+} in the vicinity of open channels (within so-called Ca^{2+} microdomains) triggers the fusion of docked and primed synaptic vesicles. A key element in exocytosis is the formation of a complex between so-called SNARE (soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein (SNAP) receptor) proteins (BOX 1) between opposing vesicular and plasma membranes[13,23]. The four-helical *trans*-SNARE bundle between synaptobrevin (a synaptic vesicle transmembrane protein) and the plasma membrane proteins synaptosome-associated protein (relative molecular mass 25K) (SNAP25) and syntaxin is thought to provide the driving force for the fusion reaction. Recent data suggest that one to three SNARE complexes[24,25] may be sufficient to overcome the activation energy for the merger of lipid bilayers. The activity of SNAREs is tightly controlled by Munc13 and Munc18, two proteins that are considered to be involved in the priming of vesicles for release[23] (FIG. 2). Microdomain Ca^{2+} is sensed by members of the synaptotagmin family of Ca^{2+}-binding C2 domain protein (FIG. 2), which may act on SNARE complexes and on the membrane itself[12,26]. There are a number of other important regulators, including Ras-related Rab proteins and complexins[27], that impose additional layers of control on the fusion process[13,23].

After membrane fusion, assembled SNARE complexes, perhaps bound to some of their regulators, may occupy the release site. The immediate fate of these SNARE complexes is unknown. However, to sustain release in the long run, SNARE complexes must be disassembled and their components, as well as other proteins, re-sorted according to their destination. Disassembly of the SNARE complex occurs through energy-dependent mechanisms by the specialized ATPase NSF and its adaptor protein, the α-soluble NSF attachment protein (α-SNAP)[7,8,13,23] (FIG. 2). Synaptobrevin, for example, is dissociated from its SNARE partners, sorted, translocated and then endocytosed. As discussed below, together these steps constitute a putative kinetic bottleneck affecting both endocytosis and subsequent release events[9] and, hence, may be rate-limiting steps in the efficiency of exocytic–endocytic coupling at a particular synapse.

Orchestrating release: presynaptic active zone scaffolds. In most synapses, neurotransmitter release preferentially occurs in a spatially defined manner from specialized release sites within the active zone in the plasma membrane[3,8,22] (BOX 1). The active zone provides the platform for rapid fusion of synaptic vesicles after Ca^{2+} influx. Associated with the intracellular face of the active zone membrane is an electron-dense cytomatrix comprising a set of large multidomain proteins, collectively referred to as the cytoplasmic matrix of the active zone[3] (CAZ; FIG. 1). Such protein scaffolds are defined by their multidomain character (linking different functionalities) and their direct or indirect

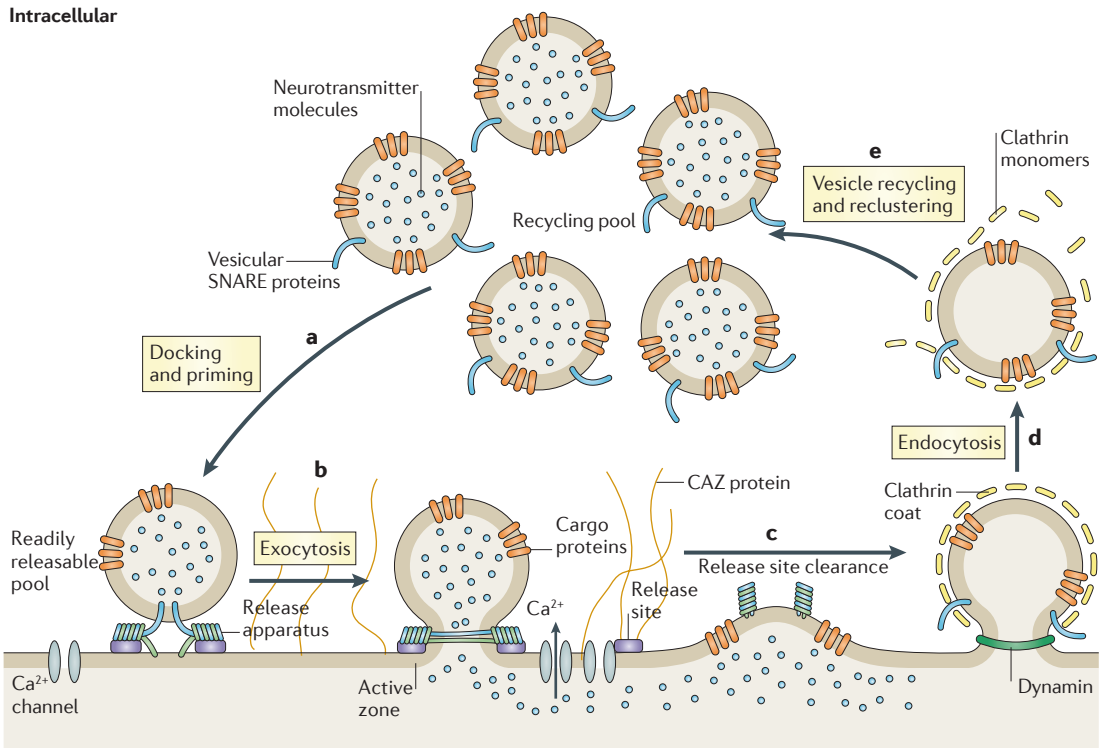

Figure 1 | **An overview of the synaptic vesicle cycle.** Docked and primed synaptic vesicles (**a**) constitute the readily releasable pool. Following Ca^{2+} influx they undergo exocytosis (**b**) and release neurotransmitter into the synaptic cleft, where these neurotransmitter molecules can activate postsynaptic receptors. Exocytosis occurs preferentially at release sites within specialized areas of the presynaptic membrane, called active zones, which are defined by their spatial proximity to voltage-activated Ca^{2+} channels and, presumably, by the presence of cytoplasmic matrix of the active zone (CAZ) scaffolding proteins. To maintain the availability of release sites for subsequent fusion reactions, release sites have to undergo clearance (**c**), an enigmatic step connecting the exocytic and endocytic limbs of the synaptic vesicle cycle. Endocytosis of synaptic vesicle membranes, including transmembrane synaptic vesicle cargo proteins, preferentially occurs within the periactive zone that surrounds the active zone area, and may be connected to it via protein–protein interactions. Synaptic vesicle endocytosis is predominantly mediated by a clathrin- and dynamin-dependent pathway involving endocytic scaffolds and specialized synaptic vesicle sorting adaptors (**d**). Following clathrin uncoating and concomitant neurotransmitter uptake, synaptic vesicles return to the recycling pool, where they undergo clustering (**e**).

association with membranes, which together provide 'smart' interaction surfaces to spatiotemporally organize protein–protein interactions (FIG. 2) or enzymatic activities. Examples of CAZ components include the giant proteins bassoon and piccolo, Rab6 interacting protein (ELKS; also known as CAST1), mammalian relatives of the *Drosophila melanogaster* protein bruchpilot (BRP), liprin and GIT family proteins, Rab3 interacting molecules (RIMs), and the SNARE regulator Munc13 (FIG. 3a). Munc13, for example, regulates efficient neurotransmitter release[28] by forming an interaction web with the active zone components piccolo, bassoon, CASTs and RIMs[28] (FIG. 3a).

The importance of the CAZ in orchestrating neurotransmitter release is further exemplified by the fact that flies with null alleles of BRP[29] show loss of the electron-dense active zone cytomatrix, a morphological defect that is correlated with impaired clustering of voltage-gated Ca^{2+} channels and with desynchronization of glutamate release. Alterations in activity-dependent synaptic vesicle exocytosis are also seen upon knockdown of the giant CAZ protein piccolo[30].

Although ultra-structure and composition of the presynaptic cytomatrix are quite variable at different synapse types, it is likely that at all synapses, CAZ components play important parts in the organization of the machinery associated with synaptic vesicle exocytosis and endocytosis, including Ca^{2+} channels (FIG. 2). As detailed below, we argue that CAZ components (FIG. 3) may be an important element in coupling exocytic synaptic vesicle fusion and clearance of release sites with endocytic retrieval of synaptic vesicle membranes near the active zone and at the surrounding periactive zone.

Closing the cycle: synaptic vesicle endocytosis and recycling. Synapses usually contain a limited number of synaptic vesicles, often less than one hundred. As synapses are usually located at a great distance from the protein synthesizing machinery in the cell body, this organization necessitates rapid local recycling of synaptic vesicle membranes within the nerve terminal[6–8] under conditions of fast neurotransmission. Physiological studies (BOX 2) on nerve terminals and sensory systems, including retinal bipolar cells[31] and the calyx of Held in the

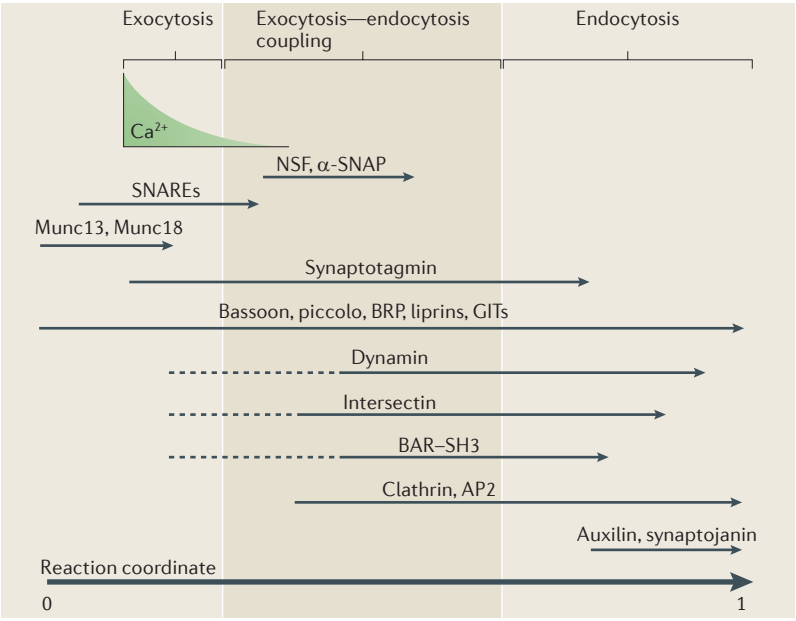

Figure 2 | **Hypothetical timing of synaptic vesicle exocytosis–endocytosis.** Exocytosis (shown in green) is initiated by high local microdomain Ca^{2+}, which drives SNARE (soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein (SNAP) receptor)-mediated membrane fusion, a process that is facilitated by factors such as Munc13, Munc18 and the Ca^{2+}-sensing protein synaptotagmin. Exocytic–endocytic coupling (middle panel) needs to clear release sites of accumulated *cis*-SNARE complexes that must then undergo NSF–α-SNAP-mediated disassembly and, subsequently, the endocytic retrieval of synaptic vesicle components, including synaptotagmin. We propose that exocytic–endocytic coupling is facilitated by cytoplasmic matrix of the active zone (CAZ) components such as bassoon, piccolo, bruchpilot (BRP), liprins and GITs, and by endocytic scaffolds including intersectin and its binding partner dynamin. Clathrin, AP2 and bin–amphiphysin–rvs (BAR)–SH3 domain proteins drive the endocytic reaction, which is terminated by synaptojanin-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) and auxilin-dependent removal of clathrin coats. Note that the lengths of the arrows do not represent the time needed to complete these reactions.

auditory brain stem[10,32], have shown that episodes of stimulation and exocytosis are followed by endocytosis, thus restoring pre-stimulus conditions.

Endocytosis occurs with two distinct time constants, with at least some of the components being highly sensitive to manipulation of intracellular Ca^{2+} concentrations[32–34]. These results indicate that multiple mechanisms are involved, but they need to be interpreted with some caution, as some of the kinetic components (for example, the rapid component in the calyx of Held) are only observed after very intense, possibly non-physiological stimulation. There are also indications that prolonged whole-cell recording (BOX 2) compromises endocytic capacity at the calyx of Held[35]. Thus, under these conditions certain aspects of endocytic cycling may be non-functional. In agreement with this, three distinct components of endocytosis have been described in adrenal chromaffin cells[36], one of which was rapidly lost in the course of an experiment in the whole-cell configuration. Hence, the control mechanisms operating in an unperturbed nerve terminal remain somewhat uncertain.

pH-sensitive pHluorin reporters (BOX 3) for exocytic–endocytic cycling of synaptic vesicles[11] are not subject to the 'washout' problem of electrophysiological measurements referred to above, but are also often used in combination with very strong stimulation (for obtaining a well-resolved signal). Data from hippocampal neurons in culture under different stimulation conditions indicate that there might be a single exponential process with a time constant of 10–15 s[37]. Endocytic rate constants in addition to potential regulation by Ca^{2+} (REF. 38) are also partially sensitive to exocytic load[39]. Under conditions of excessive non-physiological stimulation, endocytic capacity is overrun by exocytic load, resulting in the transient accumulation of synaptic vesicle proteins on the neuronal surface — a condition that may trigger bulk uptake of the presynaptic plasma membrane into vacuolar invaginations, as seen at retinal bipolar cell terminals[40] and in cultured neurons[41]. How synaptic vesicles emanate from bulk endosomes and to what degree endosomal sorting[42] contributes to synaptic vesicle recycling under physiological conditions remain matters of debate[43].

From photobleaching and proteolysis experiments it seems that a fraction of pHluorin–green fluorescent protein (GFP)-tagged synaptic vesicle proteins (BOX 4) is resident at the presynaptic membrane. These proteins are mobile and intermix with newly exocytosed synaptic vesicle proteins[44,45]. By contrast, super-resolution stimulated emission depletion (STED) microscopy (BOX 4) has suggested that newly exocytosed synaptic vesicle proteins are clustered at the plasma membrane[46]. This apparent contradiction could be explained by differences between pHluorin-tagged synaptic vesicle membrane proteins and their native endogenous counterparts[47]. Alternatively, synaptic vesicle proteins may undergo postexocytic reclustering within the periactive zone[44,45] (FIG. 4), a process that could become rate-limiting under conditions of sustained activity (see below).

Molecular analysis using genetic, biochemical and optical imaging approaches have provided strong evidence for an important, if not essential, role of clathrin- and dynamin-mediated endocytosis in synaptic vesicle reformation[11,48]. Acute chemical, sustained genetic or RNAi-mediated perturbation of clathrin[11] or dynamin function in a variety of organisms and model systems[7,48–51] causes strong defects in synaptic vesicle recycling. By contrast, elimination of other endocytic proteins in mammalian synapses has revealed surprisingly subtle effects, suggesting that many functions of endocytic factors may be redundant[6].

Recapturing of exocytosed synaptic vesicle proteins within the periactive zone is achieved by adaptor proteins including the heterotetrameric AP2 complex[52], stonin 2 (also known as stoned B)[53,54] and perhaps AP180, which serve as cargo-specific sorters of select synaptic vesicle proteins[6,55] (FIG. 2). How precisely the full complement of synaptic vesicle proteins is re-collected has remained elusive and may involve a combination of cargo-specific sorting and synaptic vesicle protein aggregation. Moving exocytosed synaptic vesicle proteins away from the release site also seems to be a crucial step in exocytic–endocytic coupling, as discussed below.

Sorting of synaptic vesicle proteins is accompanied by endocytic protein-mediated deformation of

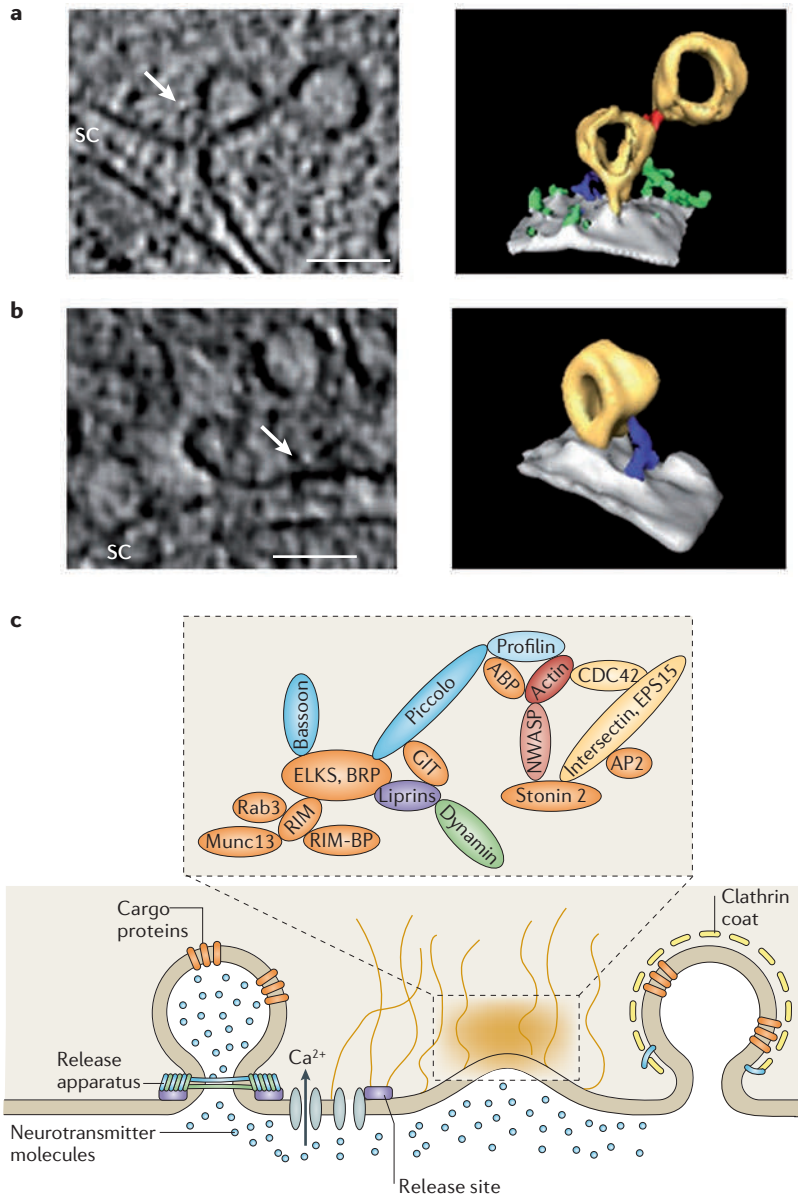

Figure 3 | **Scaffolds in exocytic–endocytic coupling.** Direct membrane contact between synaptic vesicles and the active zone. Tomographic slices that are 2.7 nm in thickness (**a**,**b**; left panels) and the corresponding direct three-dimensional rendering of the electron micrograph densities are shown (**a**,**b**; right panels). A synaptic vesicle with an open neck, which establishes continuity between the vesicular lumen and the extracellular space, and an L-shaped density close to the neck (shown by a white arrow) are seen in **a**. A synaptic vesicle making membrane contact with an invagination of the active zone and an L-shaped density close to the active zone invagination (shown by a white arrow) are seen in **b**. Synaptic vesicles are shown in yellow, synaptic vesicle connectors in red, the active zone in grey, synaptic vesicle-associated densities in blue and other active zone densities in green. The scale bars represent 50 nm. A schematic diagram depicting the multidomain protein scaffolds and their major binding partners, possibly coupling exocytosis and endocytosis, is also shown (**c**). Protein–protein interactions — which can, for example, involve piccolo — link cytoplasmic matrix of the active zone (CAZ) components to periactive zone proteins such as intersectin and epidermal growth factor receptor substrate 15 (EPS15). These proteins serve as scaffolds that connect synaptic vesicle sorting adaptors, such as stonin 2 (also known as stoned B), to the general endocytic machinery (that is, AP2) and to the actin cytoskeleton. ABP, an actin-binding protein; BRP, bruchpilot; CDC42, cell division control protein 42; ELKS, Rab6 interacting protein (also known as CAST); NWASP, neural Wiskott-Aldrich syndrome protein; RIM, a Rab3 interacting molecule; RIM-BP, RIM binding protein; SC, synaptic cleft. Parts **a** and **b** are reproduced, with permission, from REF. 106 © (2010) Rockefeller University Press.

phosphatidylinositol 4,5-bisphosphate (PIP2)-rich membranes[56]. Membrane bending mechanistically occurs by the insertion of amphipathic helices into the cytoplasmic leaflet of the plasma membrane and involves crescent-shaped F–BAR (FCH domain–bin–amphiphysin–rvs; FCH–BAR)-containing proteins such as FCHO proteins, amphiphysin, endophilin, syndapin as well as the ENTH domain of epsin[57]. The assembling clathrin coat formed from mixed pentagonal and hexagonal arrays of clathrin triskelia provides a scaffold[6,8,14,55] that serves three main purposes. First, it stabilizes the deformed membrane patch[58], thereby regulating synaptic vesicle size; second, clathrin (and/or adaptor scaffolds) concentrates synaptic vesicle cargo; and third, the assembled clathrin lattice organizes the dynamic flux of endocytic proteins needed for clathrin-coated pit maturation, dynamin-mediated fission and uncoating (FIG. 2).

A crucial part in synaptic vesicle endocytosis is played by the GTPase dynamin (FIG. 2), a mechanochemical enzyme recruited to endocytic sites by interaction with SH3 domain proteins[58,59], including intersectin[60–62], amphiphysin[63], endophilin[64,65] and syndapin[66], suggesting a tight interplay between BAR–SH3 protein-mediated membrane deformation (FIG. 2) and dynamin-catalyzed fission[58,67]. In agreement with this, dynamin 1 knockout mice exhibit a striking, activity-dependent accumulation of tubular coated endocytic intermediates[48]. Data from dynamin temperature-sensitive mutant flies (*shibire*^{ts}) suggest a possible role for dynamin that may go beyond its established role in endocytic membrane fission (see below). Following uptake of neurotransmitter, newly endocytosed synaptic vesicles are recycled to the synaptic vesicle cluster. Actin may provide a structural element for guiding synaptic vesicles back to the reserve pool[68,69], the integrity of which is intimately linked to the synaptic vesicle-associated actin binding and bundling phosphoprotein synapsin[70]. How precisely synapsin mediates synaptic vesicle clustering is unknown.

Comparatively little is known about the structural organization of the endocytic machinery in the periactive zone. Genetic studies have identified the multidomain endocytic scaffolding proteins intersectin[60–62,71] and epidermal growth factor receptor substrate 15 (EPS15)[72] as crucial for synaptic vesicle membrane retrieval and synapse development in *Drosophila melanogaster* and *Caenorhabditis elegans*. Expression levels of intersectin and EPS15 are interdependent, and the phenotypes observed on loss of either protein are nearly identical, indicating a close functional relationship between both proteins[72]. Consistent with these observations EPS15 and intersectin, together with FCHO proteins, serve as nucleators for clathrin-coated pit assembly[73]. *Drosophila melanogaster* intersectin mutants display severe defects in synaptic vesicle recycling, an accumulation of endocytic intermediates at active and periactive zones, and reduced levels of endocytic proteins[60,61]. As intersectin interacts with several endocytic proteins, including AP2, stonin 2, dynamin, the exocytic SNARE protein SNAP25, and with the actin regulatory proteins neural Wiskott-Aldrich syndrome protein (NWASP) and

Box 2 | **Functional access to exocytosis–endocytosis by electrophysiology**

Electrophysiological analysis is often used to functionally analyse synaptic transmission. However, direct access to exocytic–endocytic synaptic vesicle cycling can be hampered by the small size of presynaptic terminals. Exceptions to this are the rodent calyx of Held, a giant nerve terminal of the auditory brain stem. Presynaptic capacitance measurements of surface membrane area are effectively used to directly follow synaptic vesicle exocytosis and endocytosis[33,34,81], whereas Ca^{2+} can be optically measured and controlled experimentally by uncaging techniques. A drawback of the capacitance technique is that it measures the net change in surface area rather than measuring exocytosis and endocytosis separately. Also, it must be pointed out that most of the capacitance studies so far have been performed in the whole-cell recording mode, using patch pipettes. In this configuration, small molecules can freely diffuse between the nerve terminal and the recording pipette, potentially depleting or 'washing-out' important regulatory components, such as second messengers and small soluble proteins. The perforated-patch technique avoids this problem[114].

cell division control protein 42 (CDC42), it constitutes another potential molecular linker between synaptic vesicle exocytosis and endocytosis[62] (FIGS 2,3).

Evidence for exocytic–endocytic coupling

Fusion of synaptic vesicles with the membrane of the nerve terminal implies that the nerve terminal expands. Sustained synaptic activity would therefore lead to drastic morphological changes, unless exocytosis is tightly compensated by endocytosis. Such 'compensatory' endocytosis is indeed observed in neurons, although in most other cell types and systems endocytosis seems to be a constitutive process. Synaptic vesicle reformation at the nerve terminal is strongly activity-dependent, resulting in spatiotemporal coupling to the exocytic fusion of synaptic vesicle membranes[6,8,63,74]. Under conditions of low-level synaptic activity, endocytic intermediates — for example, clathrin-coated structures undergoing fission — have been observed close to the release site as well as at more distant, lateral sites[74,75]. The mechanisms by which exocytosis and endocytosis are functionally coupled are the subject of much controversy, possibly owing to the multiple kinetic components that are involved, each with different underlying mechanisms (see above).

A central regulatory part seems to be played by the local intracellular Ca^{2+} concentration, which rises and falls rapidly during an action potential. At concentrations well below the threshold for exocytosis, Ca^{2+} may facilitate endocytosis in model systems such as the lamprey reticulospinal synapse[74]. Recently, the synaptic vesicle-associated transmembrane protein known as flower (a proposed Ca^{2+} channel) was shown to regulate synaptic vesicle endocytosis, and thus, potentially control exocytic–endocytic coupling[76]. However, the role of Ca^{2+} may actually be supportive rather than mandatory, because endocytosis was found to be intact in hippocampal nerve terminals at resting Ca^{2+} levels after sucrose-induced release[77]. This finding suggests that some other signal or limiting resource is necessary for eliciting endocytosis.

Membrane-bound synaptic vesicle proteins (for example, synaptobrevins, synaptotagmins or synaptophysin) are possible candidates for conveying such a signal or carrying an essential structural element for vesicle formation. They have to recycle and can do so only by endocytosis. Indeed, this idea is corroborated by findings that proteins that were considered to be exclusively involved in exocytosis apparently have an additional role in synaptic vesicle endocytosis. For example, neurons lacking the exocytic SNARE protein synaptobrevin 2 show a strong defect in synaptic vesicle reformation following high sucrose-induced depletion of the RRP[77]. Furthermore, synaptic vesicle endocytosis is slowed approximately twofold or threefold in synaptotagmin 1 knockout mice[78]. Endocytic defects are also observed in mice lacking the synaptotagmin 1-associated synaptic vesicle proteins synaptic vesicle glycoprotein 2A and synaptic vesicle glycoprotein 2B[79]. Even more dramatic changes occur upon acute photoinactivation of synaptotagmin 1 at fly neuromuscular junctions, with a near complete loss of stimulus-evoked synaptopHluorin retrieval[80]. Collectively, these data suggest a crucial role of certain synaptic vesicle proteins in coupling the exocytic and endocytic limbs of the vesicle cycle.

Exocytic-endocytic coupling and short-term synaptic depression. Synaptic vesicles specifically dock to release sites at the active zone, from which rapid Ca^{2+}-triggered fusion can occur. Quantitative analysis of quantal neurotransmitter release has provided evidence that the number of release sites per active zone might be fixed[9]. These sites are likely to be located in close proximity to presynaptic Ca^{2+} channels[81]. This places the Ca^{2+} sensor of the release apparatus within Ca^{2+} nanodomains, where the concentration of Ca^{2+} is highly elevated upon Ca^{2+} channel opening. This spatial coupling may be stabilized by septin filaments[82] and is thought to be responsible for the highly synchronized release that is observed during action potentials.

Demands on availability and reuse of both release-sites and vesicles may be high, as the following example shows: data from combined electron microscopic and physiological analyses of the calyx of Held synapse suggest that there are three independent release sites per active zone[9]. Each of these sites must be used several times per second in order to support the release, which this synapse delivers during high-frequency stimulation (see REF. 9 for a review). Firing rates between 50 Hz and 300 Hz are quite 'physiological' in the auditory pathway[1]. As a consequence, the calyx of Held synapse is in a state of 'short-tem depression' during normal operation.

The standard experiment to probe short-term depression is to let the synapse rest for a few seconds and then to apply a short, high-frequency train stimulus. The first excitatory postsynaptic current (EPSC) in the resulting response will be large, as it draws from a large pool of

release-ready vesicles (most of the release sites are occupied at rest). Subsequent responses are progressively smaller, mainly owing to the decrease in the availability of release-ready vesicles. Typically, after 5 to 10 stimuli, a steady state is reached, which is characterized by a balance of synaptic vesicle consumption and synaptic vesicle resupply. The level of depression, therefore, depends on the speed of recruitment of release-ready synaptic vesicles. Another indicator of synaptic vesicle recruitment is the recovery of a single postsynaptic response, elicited at various time delays after the end of a stimulus train.

What limits the speed of such recovery? Several features of short-term synaptic depression suggest that under high-frequency stimulation, the demand on the recycling of release sites is more stringent than that on the availability of synaptic vesicles, which outnumber release sites by several orders of magnitude. Under these circumstances, clearance of release sites may actually be the rate-limiting step for release and delayed clearance may underlie the short-term depression observed[9].

A number of recent studies have suggested a role for endocytic proteins in regulating vesicle exocytosis, short-term depression and recovery from depression. Strikingly, this role seemed to be in addition to the function of these proteins in synaptic vesicle recycling. *Drosophila* mutant flies carrying a temperature-sensitive allele of dynamin (*shibire*^{ts}) at the non-permissive temperature display rapid synaptic fatigue within 20 ms of stimulation. This phenotype cannot be explained by synaptic vesicle depletion, as electron microscopy shows normal numbers of synaptic vesicles after short episodes at non-permissive temperatures[83]. Hence, dynamin might be required for short-term maintenance of the RRP of synaptic vesicles, thereby potentially coupling exocytosis and endocytosis, in agreement with ultrastructural data from mutant fly neuromuscular junctions[51].

Mechanistically, this phenotype could be explained by the accumulation of exocytosed synaptic vesicle proteins or endocytic intermediates that jam the release site, making it unavailable for further rounds of exocytosis. Consistent with this proposal, Hosoi *et al.*[34] have observed delayed vesicle recruitment following manipulation of endocytic proteins at the calyx of Held. Acute interference with dynamin function by the small molecule inhibitor dynasore, anti-dynamin antibodies, or infusion of an SH3 domain-blocking peptide, delayed vesicle recruitment and enhanced short-term depression, similar to what has been observed at the neuromuscular junction of *shibire* mutant flies. An almost identical phenotype was observed upon interference with the interaction between synaptotagmin and endocytic proteins or infusion of a proline-rich peptide derived from synaptobrevin[34].

All of these results were interpreted to reflect defects in release site clearance, which would make sites refractory for new rounds of vesicle docking and release. The data clearly indicate that the processes of exocytosis and endocytosis are mechanistically linked together at the calyx of Held, and presumably also at other synapses.

Box 3 | **Visualizing presynaptic exocytosis and endocytosis**

Labelling of synaptic vesicles is often accomplished by fluorescent styryl dyes (FM dyes), the presynaptic uptake and release of which can be monitored in living preparations in real time[115]. In FM dye experiments, the synaptic preparation is bathed in a solution containing the dye, which strongly absorbs to membranes. As synaptic vesicles open to the extracellular solution (after exocytosis), their internal membrane leaflets become labelled with the dye, and subsequent endocytosis then leads to uptake of the dye. Endocytosed synaptic vesicles remain labelled even if the preparation is thoroughly washed to remove external dye, and remaining fluorescence is therefore indicative of preceding exocytosis and endocytosis. Subsequent strong stimulation in the absence of the dye can probe whether recycled vesicles are release competent, as vesicles will release dye during new cycles. Different FM dyes exhibit distinct partitioning coefficients and wavelengths that allow for probing the system. Recently, quantum dots have been introduced as an alternative tracer to monitor fusion events[116]. A distinct advantage of such live tracers is the fact that they can be applied directly to preparations without a need for genetic manipulation or transfection. However, these assays are limited in terms of their sensitivity and temporal resolution. They can be prone to artefacts owing to non-synaptic membrane turnover contaminating the signals.

Alternatively, fusion constructs of pH-sensitive green fluorescent protein (GFP) variants with synaptic proteins (so-called pHluorins) have been successfully used as exocytic–endocytic reporters in hippocampal neurons in culture or in slice preparations from transgenic mice that stably express pHluorin reporters[38,117]. The fluorescence of these proteins is quenched while the GFP moiety resides in the low pH of the vesicle lumen, but is activated after exocytosis, when the proteins are exposed to the neutral extracellular milieu. Fusion proteins between pHluorin–GFP and various synaptic vesicle proteins have been reported[38,117]. They differ in the signal-to-noise ratios following stimulation-induced exocytosis–endocytosis, with vesicular glutamate transporter 1 (VGLUT1) pHluorin or synaptophysin pHluorin being considered superior over the more traditionally used synaptobrevin 2 pHluorin. A recently introduced variation is the pH-sensitive organic dye CypHer, which fluoresces predominantly at the acidic intralumenal pH. When coupled to antibodies directed against the lumenal domain of a synaptic vesicle protein, CypHer yields signals that are inversely correlated with those obtained from pHluorins.

Models of exocytic–endocytic coupling

In the following section we will use the information discussed above and experimental data to formulate hypothetical models of how scaffolding proteins and associated enzymatic activities may couple exocytosis and endocytosis.

These models and predictions regarding the components involved in exocytic–endocytic coupling are based on our ideas regarding the molecular steps that immediately follow synaptic vesicle fusion. These steps define the refractory period between exocytosis and endocytosis until another readily releasable vesicle is able to target an active zone release site after its use in a previous round of exocytosis. We further assume that at least the initial steps of exocytic–endocytic coupling occur in the immediate vicinity of the active zone and thus may be organized by CAZ components that serve to orchestrate multiple activities in the complex exocytic–endocytic network.

An event that must follow the exocytic reaction is the removal of postfusion *cis*-SNARE complexes that will have to leave active zone release sites (also termed 'slots') in order to prevent congestion of such sites. Monomeric SNARE proteins are required for further rounds of SNARE complex formation (FIGS 1,2) and *cis*-SNARE complex disassembly (through NSF; see above) may be a prerequisite for re-sorting of synaptic vesicle protein during endocytosis. A recent study by Ordway

Box 4 | **Models and systems to study presynaptic exocytosis and endocytosis**

Exocytosis and endocytosis of synaptic vesicles have been studied using three main techniques: light microscopy-based methods, ultrastructural analysis by electron microscopy and electrophysiological approaches. For light microscopy-based methods, specific proteins are manipulated to address their particular function in exocytosis and endocytosis, and to define discrete steps within the exocytic–endocytic cycle. In this context, genetically accessible model systems, most prominently the fruitfly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, display distinct advantages as these models allow for an unbiased genetic identification of synaptic proteins by functional screening approaches. The *Drosophila melanogaster* neuromuscular junction combines effective genetic access with a comparatively simple morphological organization that offers the possibility for postsynaptic voltage clamp recordings. Morphological and electrophysiological recordings can be paired at large synapse preparations, such as the reticulospinal axons of the lamprey[63] or the calyx of Held synapse in the auditory brain stem of mammals[34,81,113,118]. Electron microscopy is mainly used to describe individual synaptic vesicles in the synaptic context, given that the size of individual synaptic vesicles is below the resolution limit of conventional light microscopy. Electron microscopy, however, precludes live analysis and is often laborious, and retrieving molecular information depends on the availability of appropriate, specific antibodies. Some of these limitations may be bypassed by super-resolution light microscopy techniques (for example, photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM) and stimulated emission depletion microscopy (STED)), which fundamentally break the resolution limit of traditional imaging methods imposed by Abbe's law[119].

These techniques provide spatial resolution in the nanometer range and may enable the tracking of the fate of cycling synaptic vesicle membranes and of the role of protein scaffolds within the synaptic vesicle cycle in real time. Some of these techniques may be adapted for the use of switchable optical tracers, which would enable the visualization of the distribution of newly exocytosed vesicle proteins immediately after their exocytic insertion into the presynaptic membrane.

and colleagues[84], however, showed that acute block of NSF did not cause increased short-term depression but was instead important for sustained vesicle release over extended periods of time. These data suggest that although NSF is required to recycle SNARE proteins, it is dispensable for the clearance of release sites.

Hypothetical models for release site clearance. Complete fusion of synaptic vesicle membranes will result in the accumulation of synaptic vesicle cargo proteins at the release site, thereby causing a functional block of the previously used active zone slot. How these newly exocytosed synaptic vesicle proteins are removed from the release site is unknown. Clearance of the active zone slot could be achieved by rapid lateral diffusion of patches of synaptic vesicle proteins towards the periactive zone. Cytoskeletal elements — in particular, local actin dynamics and perhaps also septins[82] — might confer directionality to this process (FIG. 4a).

Several proteins of the exocytic–endocytic machinery interact with, or regulate, components of the actin cytoskeleton[3,14,66,68,69]. For example, the large multidomain CAZ component piccolo associates with profilin, a proline-rich protein that binds to actin monomers and facilitates ADP to ATP exchange. Piccolo also interacts with actin-binding protein 1 (ABP1; FIG. 3c), a factor linking dynamin-dependent endocytosis to the actin cytoskeleton[28,85]. Furthermore, the piccolo binding partner GIT associates with liprin-α (also known as SYD2), another CAZ component (FIG. 3c), and with the actin-associated protein Rho guanine nucleotide exchange factor 7 (also known as β-PIX).

Strong links with the actin polymerizing machinery are also seen for the endocytic scaffolding protein intersectin, which is a CDC42 guanine nucleotide exchange factor and binding partner of NWASP[62] of dynamin[59]. This network of exocytic–endocytic scaffolding proteins (FIG. 3c) might direct the assembly of short actin filaments, which then facilitate and/or organize the lateral diffusion of newly exocytosed patches of synaptic vesicle proteins towards endocytic sites at the rim of the active zone membrane (FIG. 4a). Such a scenario is supported by the fact that several of these proteins display multiple interactions with the endocytic machinery required for synaptic vesicle protein sorting. For example, intersectin undergoes complex formation with early acting factors including FCHOs[73] and EPS15, and with the sorting adaptors AP2 (REF. 71) and stonin 2 (FIG. 3c). Hence, the rate of release site clearance might be determined by the speed with which such directed diffusion of synaptic vesicle components away from the fusion site occurs.

Conflicting light-microscopic data regarding the mobility and state of clustering of newly exocytosed proteins[44–47] (FIG. 4b) may be explained by a model assuming that synaptic vesicle proteins initially decluster, but then undergo rapid reclustering (FIG. 4b), perhaps aided by endocytic sorting adaptors such as AP2 and stonin 2 (REFS 53–55), which are enriched near active zone membranes through interactions with CAZ components or associated bridging factors. Time-resolved experiments using super-resolution light microscopy paired with live imaging or switchable optical tracers might help to resolve this issue by visualizing the distribution of newly exocytosed vesicle proteins immediately after their exocytic insertion into the active zone membrane.

As mentioned above, acute or genetic interference with dynamin function at *Drosophila melanogaster* flight muscles[83] or at the calyx of Held synapse[34] provokes short-term depression, a phenotype that synaptic vesicle depletion can probably not account for. How can these findings be explained? Although the role of dynamin in catalyzing scission of endocytic vesicles is undisputed, it is conceivable that dynamin executes an additional function in exocytic–endocytic coupling. Common to both roles may be the ability of dynamin to deform and remodel membranes — a property of all dynamin family proteins studied so far — perhaps in conjunction with

Super-resolution light microscopic techniques
Forms of light microscopic technique that achieve spatial resolution of 50 to 100 nm, beyond the limit set by diffraction; they include stimulated emission depletion microscopy (STED), photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM).

its SH3 domain-binding partners. Although a precise mechanism has not yet emerged, several tentative connections between dynamin and Ca^{2+}-dependent exocytic–endocytic coupling have been uncovered. Dynamin 1 knockout mice display severe defects in synaptic vesicle endocytosis during stimulus-driven Ca^{2+} influx[48].

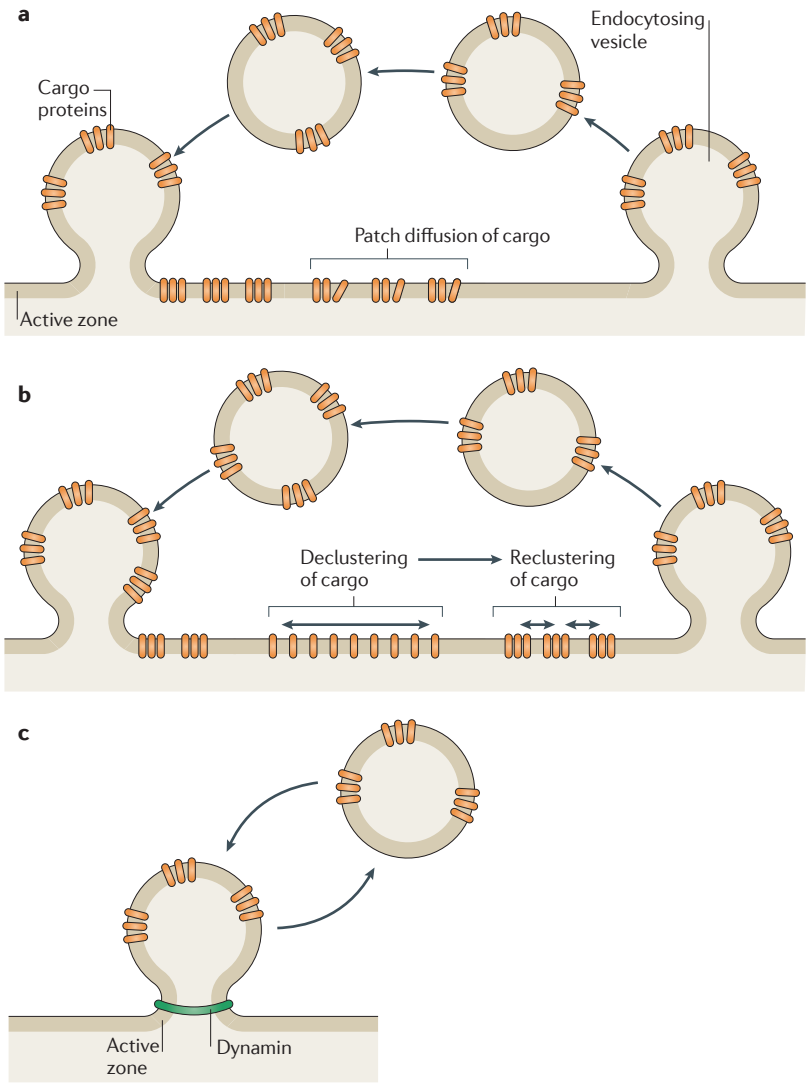

Figure 4 | **Hypothetical models for exocytic–endocytic coupling.** Following exocytosis and disassembly of *cis*-SNARE (soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein (SNAP) receptor) complexes (not shown), clustered synaptic vesicle proteins (cargo proteins) may be transported or diffuse from the active zone towards the periactive zone, where endocytic retrieval occurs. Recycled synaptic vesicles are then returned to the synaptic vesicle cluster for a new round of exocytosis and endocytosis. According to this model, exocytosis and endocytosis are coupled through the rate-limiting clearance of refractory release sites that are jammed with exocytosed synaptic vesicle membranes (**a**). An alternative scenario that is similar to that depicted in **a**, but in which exocytosed synaptic vesicle proteins are initially declustered and subsequently recluster within or near the periactive zone, is also shown. According to this model, newly exocytosed synaptic vesicle proteins may intermix with a pre-existing pool of surface-stranded synaptic vesicle proteins that was not retrieved during the previous exocytic–endocytic cycle (**b**). Exocytic–endocytic coupling could be achieved by the direct dynamin-mediated retrieval of synaptic vesicles at or near the exocytic site. This might be achieved through transient fusion pore opening and closure (kiss-and-run) or through the formation of coated buds containing clathrin, dynamin and other endocytic proteins (**c**).

The dynamin-binding BAR–SH3 domain protein endophilin has been postulated to interact with presynaptic Ca^{2+} channels[86], potentially linking Ca^{2+} influx to exocytic–endocytic activity. Many dynamin-associated SH3 domain proteins, including intersectin[62] and ABP1, also interact with — and regulate — components of the actin cytoskeleton and of the CAZ (FIG. 3c), such as piccolo[85]. Tentative evidence also suggests putative Ca^{2+}-dependent interactions between dynamin and calcineurin[87,88], and between dynamin and the synaptic vesicle protein synaptophysin[89]. Based on these data it is possible to speculate that dynamin may directly or indirectly contribute to the clearance of release sites at or near the active zone, perhaps through membrane fission, regulation of fusion pore collapse, actin-based processes[58] or interactions with active zone proteins (FIG. 4c). However, it also remains possible that a subpool of dynamin is dedicated to fast endocytosis near the active zone and that loss of this pool causes a retrograde jamming of release sites. Further experiments are needed to differentiate between these possibilities.

The above models remain speculative, yet, clearly are non-exclusive. It is certainly conceivable that several of the reactions and components described above might cooperate to make previously used release sites re-available.

Calcium regulation. A tight coupling between exocytosis and endocytosis is seen in fast synapses, where the geometric association between synaptic vesicles and Ca^{2+} channels is crucial. Several lines of evidence underscore an important role for Ca^{2+} in release site clearance and synaptic vesicle endocytosis[90–92]. Indeed, pharmacological manipulations at the *Drosophila melanogaster* neuromuscular junction[93,94] imply the existence of a specific Ca^{2+} channel for synaptic vesicle endocytosis. Whether this channel is identical to the recently identified *Drosophila melanogaster* synaptic vesicle protein known as flower is not yet proven[76]. Differential sensitivity to Ca^{2+} buffers suggests that local microdomain Ca^{2+} created by multiple channels also underlies slow endocytosis at the calyx of Held[34,95].

Data from various mammalian synapses indicate that calmodulin is a major effector of presynaptic Ca^{2+} during synaptic vesicle recycling[96]. Calmodulin also mediates rapid recruitment of fast-releasing synaptic vesicles at the calyx of Held[97,98], further underlining the intimate connection between presynaptic Ca^{2+}, release site clearance and endocytosis. Other Ca^{2+}-binding proteins, such as calcineurin[87,88,99,100], synaptotagmins, complexins or Munc13 (REFS 12,101) might be additional Ca^{2+} effectors facilitating exocytic–endocytic coupling.

Connecting the connectors: the potential role of active zone scaffolds. Release sites within the active zone are thought to be characterized by the spatial proximity of readily releasable vesicles and presynaptic Ca^{2+} channels[81,102]. Genetic and morphological data from invertebrate synapses, in particular the *Drosophila melanogaster* neuromuscular junction indicate that, architecturally, such geometrical arrangements involve giant CAZ components such as RIMs, piccolo, bassoon and BRP[103], which are large enough to

bridge multiple presynaptic microdomains over distances of up to several hundreds of nanometers. By associating with exocytic–endocytic proteins[3], with components of the actin cytoskeleton such as ABP1 and profilin[28,85], and with each other, these factors may assemble into a matrix that provides attachment sites for the spatiotemporally directed movement of synaptic vesicle membranes between functionally distinct membrane domains (FIG. 3c). Consistent with this idea, reduced clustering of reserve pool synaptic vesicles has been observed in piccolo–bassoon double knockdown neurons[104], perhaps occurring through regulation of synapsin dynamics[30].

How precisely synaptic vesicles are tethered to the CAZ remains largely unknown. Small filaments that tether docked synaptic vesicles to the plasma membrane and to CAZ filaments have been observed by electron microscopy[105]. Cryoelectron tomography revealed that docked synaptic vesicles did not contact the active zone membrane directly but were linked to it by tethers of different lengths[106]. Long tethers (>5 nm) may correspond to CAZ components, whereas short tethers seem to depend on SNARE complex formation, as indicated by their sensitivity to tetanus toxin. The fact that these tethers undergo activity-dependent structural rearrangements (FIGS 3a,b)[106] might suggest a role in synaptic vesicle dynamics.

The identification and characterization of such tethers and filaments, and their relationship to CAZ components, awaits further analysis and is hampered by the fact that active zone cytomatrix components largely resist detergent extraction. Genetic analysis of presynaptic scaffolds has also turned out to be difficult because of the complex (broad) phenotypes resulting from null alleles. For example, deletion of the active zone component BRP in *Drosophila melanogaster* causes a severe loss of active zone dense bodies (T-bars) and a concomitant declustering of presynaptic Ca^{2+} channels, provoking a severe deficit in baseline synaptic vesicle release[29]. A recent study[107] identified a hypomorphic allele termed brp^{nude}, lacking merely the last 17 amino acids (that is, <1% of all residues) of BRP. In brp^{nude} flies, electron-dense T-bars representing the CAZ were properly shaped, but completely lacked the synaptic vesicles normally associated with it. Although basal glutamate release was unchanged, paired-pulse stimulation provoked a severe depression. Furthermore, rapid recovery following sustained release was slowed down[107]. These results causally link the tethering of vesicles at the active zone cytomatrix to synaptic depression and are consistent with a role of BRP in exocytic–endocytic coupling.

Clearly, the analysis of active zone components and their functional relationship with exocytic–endocytic coupling at different types of synapses is far from complete. Combined genetic, physiological, biochemical and morphological approaches, including the tailored interference with select interactions within the CAZ network, will be required to further our understanding of how presynaptic active zone scaffolds may direct synaptic vesicle cycling.

Conclusions and perspectives

Of the many tasks that nerve terminals have to accomplish, as discussed above, four require a tight spatiotemporal coordination of the elements of the synaptic vesicle cycle: first, delivery of synaptic vesicles to release sites must cope with the high demand during periods of intense synaptic activity; second, precise timing of transmitter release requires close proximity between Ca^{2+} channels and release-ready vesicles; third, high throughput of synaptic vesicles at release sites calls for effective mechanisms of site clearance that preserve the integrity of the release site and leave them free for synaptic vesicle docking; and fourth, sorting of vesicular components from those of the plasma membrane has to be achieved before endocytic reformation of synaptic vesicles within the periactive zone. These tasks require a complex set of proteins, some of which serve as structural scaffolds for the spatiotemporal coordination of these processes. Other multidomain proteins seem to serve as 'functional scaffolds' that interact with several functional and regulatory components, thereby coordinating their actions within a multiprotein functional unit.

At least two aspects of exocytic–endocytic coupling that remain poorly understood are the presumably directed movement of synaptic vesicle components away from the release site and the tight control of membrane surface area through balanced exocytic–endocytosis. At present, we can only speculate about the scaffolds that operate in release site clearance. One possible candidate for this might be intersectin, which functionally links exocytosis and endocytosis with actin polymerization at the rim of the active zone. Assembly of actin filaments may provide directionality to the process. Equally unknown are the regulatory mechanisms that keep exocytosis and endocytosis in balance. Although local intracellular Ca^{2+} is probably involved, it is tempting to postulate the existence of a 'checkpoint' that serves to ensure proper loading of endocytic intermediates with different synaptic vesicle proteins of the correct stoichiometry. In this scenario, exocytosis and endocytosis are kept in balance by the availability of synaptic vesicle proteins, which are delivered by exocytosis.

What might be the signal carriers for such a checkpoint? Membrane-integral synaptic vesicle proteins appear as candidates. It might be postulated that each element within the budding endocytic vesicle, such as a clathrin triskelion, an AP2 complex or a stonin 2 protein, pairs with a particular synaptic vesicle protein at a defined stoichiometry during assembly. It is also possible that synaptic vesicle endocytosis is controlled by the limited availability of a subset of endocytic proteins. Membrane bending is achieved by BAR domain proteins in cooperation with the assembling clathrin coat, which is required for the stabilization of nascent membrane buds. In order for this mechanism to keep the balance, BAR domain proteins would, at least in part, have to become available through exocytosis. Recent data regarding endophilin function in *C. elegans* lend support for such a scenario[108].

Clearly, more work is required to fully define the network of interactions between the endocytic machinery and their synaptic vesicle cargo. The working models suggested by current data and discussed here may guide future experiments aimed at understanding this important aspect of brain function.

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Acknowledgements
Work in the authors' laboratories was supported by grants from the German Research Foundation (Deutsche Forschungsgemeinschaft; DFG).

Competing interests statement
The authors declare no competing financial interests.

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