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Review

The presynaptic cytomatrix of brain synapses

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Abstract. Synapses are principal sites for communication between neurons via chemical messengers called neurotransmitters. Neurotransmitters are released from presynaptic nerve terminals at the active zone, a restricted area of the cell membrane situated exactly opposite to the postsynaptic neurotransmitter reception apparatus. At the active zone neurotransmitter-containing synaptic vesicles (SVs) dock, fuse, release their content and are recycled in a strictly regulated manner. The cytoskeletal matrix at the active zone (CAZ) is thought to play an essential role in the organization of this SV cycle. Several multi-domain cytoskeleton-associated proteins, including RIM, Bassoon, Piccolo/Aczonin and Munc-13, have been identified, which are specifically localized at the active zone and thus are putative molecular components of the CAZ. This review will summarize our present knowledge about the structure and function of these CAZ-specific proteins. Moreover, we will review our present view of how the exocytotic and endocytic machineries at the site of neurotransmitter release are linked to and organized by the presynaptic cytoskeleton. Finally, we will summarize recent progress that has been made in understanding how active zones are assembled during nervous system development.

Key words. Active zone; Bassoon; CAZ; endocytosis; exocytosis; neurotransmitter release; Piccolo; synapse; synaptic vesicle cycle.

Introduction

Chemical synapses are key structures for communication between nerve cells and their target cells, i.e. other neurons, muscle cells or gland cells. In the human brain, for example, about 10^{15} synaptic contacts interconnect the 10^{10}–10^{11} nerve cells. Synapses are complex asymmetric cell-cell contact sites formed by the axon terminal membrane of the presynaptic neuron and the plasmalemma of the postsynaptic cell. Within the presynaptic nerve terminal classical neurotransmitters, e.g. glutamate, acetylcholine, γ-amino-butyric acid (GABA) or glycine, are stored in clear membraneous vesicles of about 50 nm diameter, named synaptic vesicles (SVs). In response to presynaptic action potentials neurotransmitter is rapidly secreted into the synaptic cleft, diffuses across the cleft and activates neurotransmitter receptors in the postsynaptic membrane. Transmitter release is restricted to the active zone, an area of the axon terminal membrane that is precisely aligned with the postsynaptic neurotransmitter reception apparatus.

The mechanisms of regulated neurotransmitter release have been studied in detail and were reviewed on several occasions (e.g. [1–4]). SVs dock at the active zone and become fusion competent in a multi-step process. Ca^{2+} ions, which enter the nerve terminal through voltage-gated Ca^{2+} channels in response to action potentials,

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trigger rapid exocytosis of fusion-competent vesicles. Thereafter SVs are rapidly retrieved by clathrin-dependent endocytosis, recycled and refilled with neurotransmitter (for review, see [5–9]). Cytoskeletal elements have multiple functions in the organization of presynaptic nerve terminals. A specific cytoskeletal matrix (cytomatrix) is assembled at the active zone (CAZ). The CAZ is thought to be involved in defining the neurotransmitter release site during development, anchoring and localizing presynaptic membrane proteins, including cell adhesion molecules and Ca^{2+} channels, and in organizing the exocytic and endocytic machineries at the transmitter release site. Moreover, microfilaments and their associated proteins, like the synapsins, have been implicated in the spatial and functional organization of individual steps of the SV cycle. Here we will first survey the ultrastructural and functional organization of presynaptic nerve terminals, then review our present knowledge about the molecular composition of the CAZ and discuss what is known about the role of the presynaptic cytoskeleton in the organization of the SV cycle. Finally, we will summarize recent work on how the CAZ is assembled during development.

Ultrastructural and functional organization of the presynaptic nerve terminal

Various structural and functional compartments can be discriminated in axon terminals of conventional central nervous system (CNS) synapses (fig. 1). These include the active zone of neurotransmitter release, the perisynaptic plasma membrane, the SV domain and a mitochondrial domain.

The synaptic active zone

The active zone is defined as the partition of the presynaptic plasma membrane where regulated neurotransmitter release takes place. At the ultrastructural level, the active zone plasma membrane is characterized by an electron-dense projection similar to the postsynaptic density (PSD) at the transmitter reception side [10, 11]. Like the PSD, the presynaptic dense projection is thought to constitute a derivative of the cortical cytoskeleton (i.e. the actin-based cytoskeletal meshwork attached to the plasma membrane of eukaryotic cells). In the electron microscope (EM) filamentous material surrounding docked vesicles is assembled at the presynaptic membrane [12–14]. Filamentous strands originate at the active zone membrane and extend into the nerve terminal (fig. 2; [6, 15]). This cytomatrix was suggested to represent the machinery which guides SVs to the plasma membrane [6]. Moreover, the CAZ may harbor and tie together the proteins necessary for regulated exocytosis and endocytosis. It is expected to be involved in the spatial organization of the exocytotic and endocytic machineries relative to each other and in the linkage of presynaptic plasma membrane proteins, e.g. voltage-gated Ca^{2+} channels or cell adhesion molecules.

Several ultrastructural studies suggested the existence of a regularly arranged presynaptic dense projection, the hexagonal presynaptic grid, with small circular depressions (for review, see [10]). These concavities are thought to serve as docking sites for SVs. A more recent EM study described a fraction of SVs encased by polyhedral protein cages, which may mediate the association of these vesicles with the presynaptic grid [16].

The perisynaptic plasma membrane

In contrast to clear SVs, neuropeptide-containing large dense core vesicles release their contents from the perisynaptic plasma membrane laterally to the active zone [17]. The protein machinery involved in exocytosis of dense core vesicles appears to differ from that mediating SV fusion [18]. Large vesicles, probably corresponding to the neuropeptide-containing dense core granules, were observed preferentially associated with the mitochondrial domain rather than with the SV domain [19]. These large vesicles are linked to microtubules.

Outside of the active zone the axon terminal plasma membrane makes additional cell-cell contacts with the postsynaptic neuron, e.g. at the vesicle-free transition zone and at adhesion junctions (puncta adhaerentia) [10, 20]. Both junctions are characterized by electron-dense material on either side of the contact site, presumably representing specializations of the submembraneous cytoskeleton. The protein composition of these dense projections is essentially unknown. The adhesion junctions contain classical cadherins associated with catenins at their intracellular side [21]. From the adhesions junctions filaments spread out to mitochondria and smooth endoplasmic reticulum [20].

The synaptic vesicle domain

Conventional presynaptic nerve terminals in the mammalian brain contain between 30 and several hundred SVs [14, 16, 22, 23]. Complex boutons with multiple postsynaptic contacts, for example mossy fiber endings in the cerebellum or the hippocampus, may accumulate several thousands of SVs. The SVs of a synaptic bouton can be divided into three functionally different pools [24]. The release-ready pool contains the fusion-competent vesicles docked at the active zone plasma membrane. The reserve pool consists of SVs that are associated with synapsins and are more than 100–200

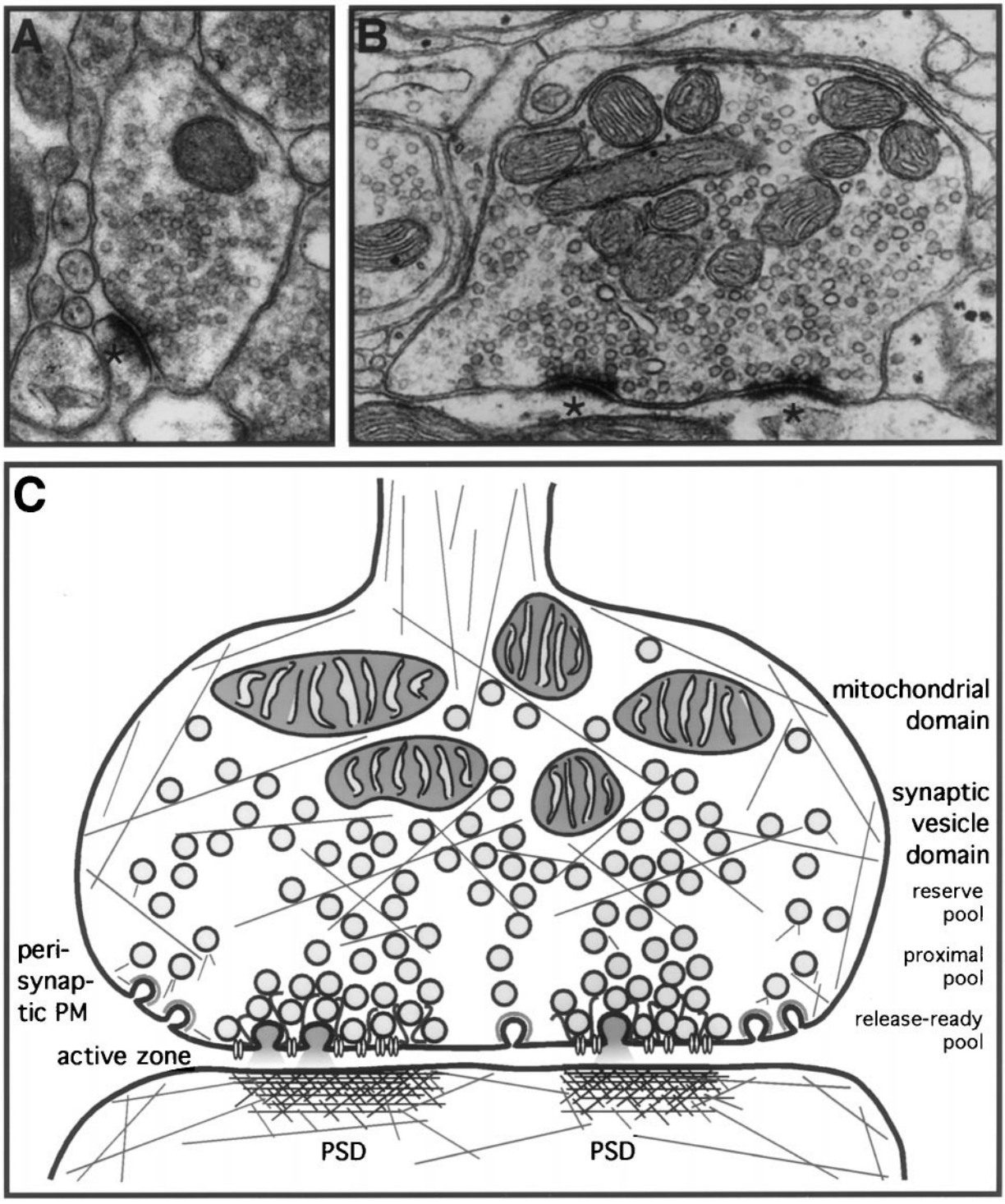

Figure 1. Ultrastructural organization of presynaptic nerve terminals. (*A*) Nerve terminal of an excitatory synapse in the human hippocampus. (*B*) Another example of a vertebrate CNS synaptic terminal with two active zones and a prominent mitochondrial domain. Postsynaptic dense projections opposite to the active zone in *A* and *B* are indicated by asterisks. (*C*) Schematic illustration of the structural organization of a presynaptic nerve terminal (for details, see text). PM, plasma membrane. Electron photomicrographs were provided by Werner Zuschratter, Magdeburg (*A*) and John E. Heuser, St. Louis (*B*, reprinted from http://www.heuserlab.wustl.edu/SynapseLink.html by permission of Dr John E. Heuser).

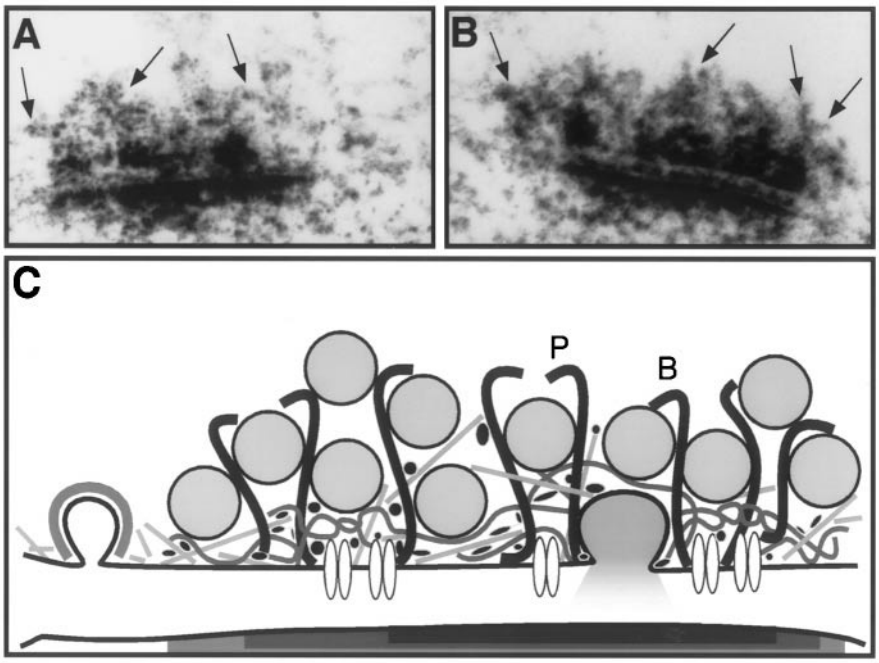

Figure 2. The cytoskeletal matrix assembled at the active zone (CAZ) of neurotransmitter release. (*A*, *B*) Visualization of the filamentous cytomatrix overlapping with the proximal SV pool. The electron micrograph shows a synaptic junction stained with phosphotungstic acid (by courtesy of Jenny Gustafsson and Lennart Brodin, Stockholm). SVs are not visible with this staining method. Arrows depict filamentous material at the presynaptic plasma membrane, some of which may be assembled from Bassoon and Piccolo [6]. (*C*) Drawing of the putative organization of the CAZ, including proximal and readily releasable pools of SVs. Bassoon (B) and Piccolo/Aczonin (P) are thought to be essential scaffolding protein of the CAZ.

nm remote to the active zone [6, 25–27]. The proximal pool, situated between the readily releasable and the reserve pools, includes two to four vesicle layers, which are thought to be synapsin-independent and may be associated with filamentous structures of the CAZ [6, 24]. SVs of the reserve pool are embedded in a filamentous meshwork of cytoskeletal elements. They are linked to each other, to filaments or to both [12–14, 19]. The linkage between the cytoskeleton, mainly actin filaments, and SVs is thought to be mediated by synapsins [13, 26]. While the release-ready and the proximal SV pools are small and directly on track to be released, the function of the reserve pool may be to replenish the former pools and to allow a synapse to operate over a wide range of activity levels.

The mitochondrial domain

Mitochondria are commonly found in presynaptic nerve terminals [10, 14, 19]. Depending on synapse type and activity pattern, the mitochondrial pool may vary in size and shape of individual mitochondria (e.g. [28]). In addition to ATP provision as energy supply and regulation of the SV cycle, mitochondria may play a role in synaptic Ca^{2+} homeostasis and neurotransmitter synthesis [29–32]. EM studies suggest that microtubules contact synaptic mitochondria either directly or via filamenteous strands [19, 28]. Moreover, an association of the motor protein kinesin with mitochondria has been reported [33].

Protein components of the CAZ

Recent studies have identified four cytomatrix proteins that are specifically localized at the active zone, i.e. Munc13-1 [34, 35], the Rab3-interacting molecule RIM [36], Piccolo/Aczonin [37–39] and Bassoon [40, 41]. In addition, a number of cytoskeletal and cytoskeleton-associated proteins are present in the CAZ, the distribution of which is not restricted to the presynapse. These include classical cytoskeletal proteins, like fodrin (or brain spectrin), actin and myosin [13, 14], as well as members of the family of membrane-associated guanylate kinase homologs (MAGuKs), like SAP90/PSD-95 [42, 43], SAP97 [44] and CASK/LIN-2 [45, 46].

Munc13

Munc13-1 is a mammalian homolog of a phorbol ester/diacylglycerol (DAG)-binding protein encoded by the

unc13 gene from the nematode *Caenorhabditis elegans* [34, 47, 48]. Mutations in *unc-13* cause diverse defects in the nervous system of the worm and a severe uncoordinated phenotype. Munc13-1 immunoreactivity is highly enriched in the vicinity of active zones of rat brain synapses. However, it is not present at all synapses examined in the hippocampus and the cerebral cortex [35]. Munc13-1 is an $\sim$200-kDa protein that contains one C1 and three C2 domains. C2 domains mediate phospholipid-dependent Ca^{2+} binding and were initially identified in protein kinase C (PKC) and the presynaptic Ca^{2+} sensor protein synaptotagmin [49]. The C1 domain of Munc13-1, like that of PKC, binds phorbol ester and DAG and is involved in phorbol ester-induced membrane association of the molecule [35]. In mammals, Munc13-1 has at least two additional isoforms, Munc13-2 and Munc13-3, encoded by paralogous genes [34]. There is convincing evidence that Munc13-1 as well as its *C. elegans* and *Drosophila* homologs are involved in SV exocytosis (see below).

RIMs

Another CAZ-specific protein is RIM1, which has been identified as an interacting molecule of the small GTPase Rab3 [36]. RIM1 is a 180-kDa protein; it contains an N-terminal double Zn^{2+} finger domain, a PDZ domain and two C2 domains. The Zn^{2+} finger is closely related to that of rabphilin. Both molecules bind Rab3 with their Zn^{2+} fingers merely in a GTP-complexed form [36, 50]. Evidence attained from transfection experiments in PC12 cells suggests that RIM1 may be a Rab3a-dependent regulator of SV exocytosis [36].

Recently, a second member of the RIM family, RIM2, was identified, which is highly homologous to RIM1 and also expressed primarily in the brain [51]. RIM2 appears to be identical with Oboe, which initially was identified in sequence tag databases based on its sequence homology to RIM1 [24, 38]. Interestingly, a splice variant of RIM2 produces a smaller protein, called NIM2, which contains a unique N-terminal sequence and the C-terminal C2 domain. Moreover, a paralogous molecule of NIM2, i.e. NIM3, has been identified [51]. In PC12 cells, NIM3 has a stimulatory effect on Ca^{2+}-dependent exocytosis.

In addition to Rab3, RIM1 binds a new family of binding proteins, RIM-BPs [51]. The binding site for RIM-BPs on RIM1 is proline rich and situated between the two C2 domains. RIM-BPs are related to PRAX1, a 220–250-kDa protein interacting with the cytoplasmic domain of peripheral benzodiazepine receptor [52]. These proteins contain three fibronectin type III and three Src homology 3 (SH3) domains. The middle SH3 domain of RIM-BPs binds RIM. At least RIM-BP1 appears specifically expressed in the brain [51].

Bassoon and Piccolo/Aczonin

Bassoon and Piccolo have been identified by a screen for novel synaptic junctional proteins from rat brain [53]. They are very large proteins of 420 kDa and 530 kDa, respectively. Because of their highly specific localization near the site of neurotransmitter release, these proteins were postulated to be members of the ensemble of proteins orchestrating events at the active zone (figs 3 and 4; [37, 38, 40]). Aczonin, which has been analyzed in chick and mouse [39], is orthologous to Piccolo. Bassoon and Piccolo occur at excitatory and inhibitory CNS synapses, but appear to be absent from cholinergic synapses, including neuromuscular junctions [37, 38, 40, 41, 54]. Moreover, Bassoon is absent from release sites of dense core vesicles [18]. In primary cultures of hippocampal neurons Bassoon and Piccolo colocalize at the vast majority of synapses [38, 40] starting from early stages of synaptogenesis [55]. Immunogold localization studies with antibodies directed against a 250-amino-acid-long region C-terminal of the second double Zn^{2+} finger (Ab7f) suggest that the molecule is localized at the active zone in a regular array [18, 54]. Gold labeling is found about one vesicle diameter away from the presynaptic plasmalemma (fig. 3).

Bassoon and Piccolo/Aczonin are related proteins. In their N-terminal region, they contain two double Zn^{2+} finger domains, which display limited homology to those of rabphilin and the RIMs. However, unlike rabphilin and RIM, the Zn^{2+} fingers of Piccolo and Bassoon do not bind Rab3A [38]. Overall, Bassoon and Piccolo share 10 regions of high homology, including the two Zn^{2+} fingers and three potential coiled-coil-forming regions [38]. At the C-terminus the two molecules diverge. Whereas Bassoon contains a region with limited homology to synapsin [56], Piccolo/Aczonin contains a PDZ and two C2 domains [38, 39]. The second C2 domain appears to be subject to alternative splicing [39].

Rodent Bassoon, but not Piccolo, contains stretches of glutamine residues encoded by CAG repeats in its C-terminal region [40]. Abnormal elongation of CAG repeats is involved in late onset neurodegenerative disorders, like Huntington's disease or various spinocerebellar ataxias [57]. Human Bassoon, however, carries only four of these repeats, and it is unlikely that abnormal expansion of CAG repeats occurs in humans [56]. On the other hand, transcript levels of ZNF231, which is identical with human Bassoon, have been found to be elevated in cerebella of patients with multiple systemic atrophy [58]. The medical relevance of this finding is currently unclear.

Bassoon and Piccolo/Aczonin are tightly associated with synaptic junctional protein preparations ('PSD' fraction) [37, 39, 40]. Together with their ultrastructural localization this argues that they are integral compo-

nents of the CAZ. However, a fraction of Bassoon and Piccolo isolated from the embryonic brain [55, 255, R. Zhai, H. V. Friedman, C. Cases-Langhoff et al., submitted] as well as a minor fraction of Bassoon isolated from adolescent rat brain [59] are associated with membrane-bounded organelles.

For Piccolo/Aczonin two potential interacting partners have been identified. The Zn^{2+} fingers of Piccolo bind the prenylated Rab acceptor protein PRA1 [38], which by itself can interact with the small GTPases Rab3 and Rab5 and with VAMP2/synaptobrevin 2, an integral SV membrane protein involved in formation of the SNARE complex during exocytosis ([60], see below for details). The second binding partner identified for Piccolo/Aczonin is profilin, which has been shown to bind to a polyproline stretch in the center of Aczonin in vitro [39]. Profilins are small G-actin-binding proteins which are involved in the regulation of the dynamics of the actin cytoskeleton and have been implicated in multiple cellular processes, including endocytosis (see below). Moreover, both Piccolo and Bassoon contain multiple additional proline-rich sequences, some of which may act as binding sites for the various SH3-domain proteins involved in endocytosis, including endophilins, amphiphysins, syndapins or intersectins (see below for details). All together, Bassoon and Piccolo/Aczonin are likely to be scaffolding proteins of the CAZ that may serve in assembling the exocytic and endocytic machinery for neurotransmitter release.

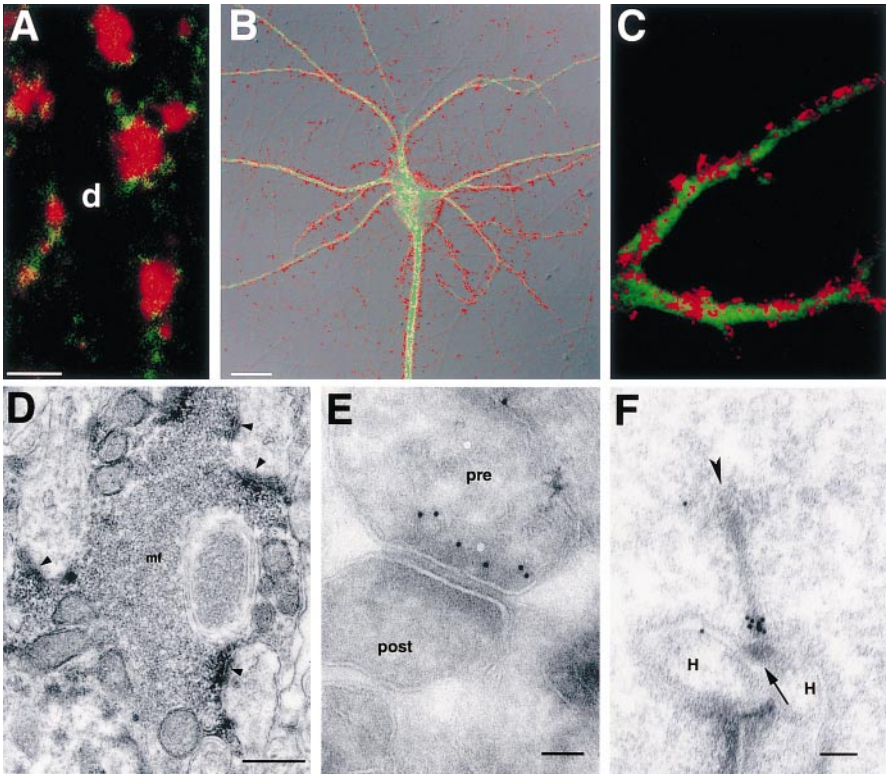

Figure 3. Localization of Bassoon at various types of synapses. (*A*) Confocal image showing immunofluorescence for Bassoon (green) and Synapsin I (red) at synapses contacting a dendrite (d) in the hippocampal CA3 region of rat brain. Scale bar, 2 μm. (*B*, *C*) Double-labeled images showing immunofluorescence for Bassoon (red) and the dendritic marker MAP2 (green) in primary cultured hippocampal neurons of the rat. In (*B*) a Nomarski image was aquired simultaneously. Photomicrographs by courtesy of Werner Zuschratter, Magdeburg, and Stefan Kindler, Hamburg. (*D*) Electron photomicrograph of a CA3 mossy fiber terminal (mf) from rat brain. Immunoreactivity is highly concentrated at active zones, opposite to PSDs (arrowheads). Scale bar, 500 nm. (*E*) Immunogold localization of Bassoon in an ultrathin cryosection of a CA1 synapse from rat brain (pre, presynaptic terminal; post, postsynaptic compartment). Scale bar, 100 nm (by courtesy of Karin Richter, Magdeburg; reprinted from the 1998/99 research report of the Leibniz Institute for Neurobiology). (*F*) Immunogold localization of Bassoon at a rod photoreceptor ribbon synapse of the rabbit retina (H, postsynaptic processes of horizontal cells). The arrowhead marks the tip, the arrow the arcifrom density at the base of the ribbon. Scale bar, 100 nm. Panels *A* and *D* were reproduced from [40] by copyright permission of the Rockefeller University Press; panel *F* was reproduced from [54] by copyright permission of Blackwell Science Ltd.

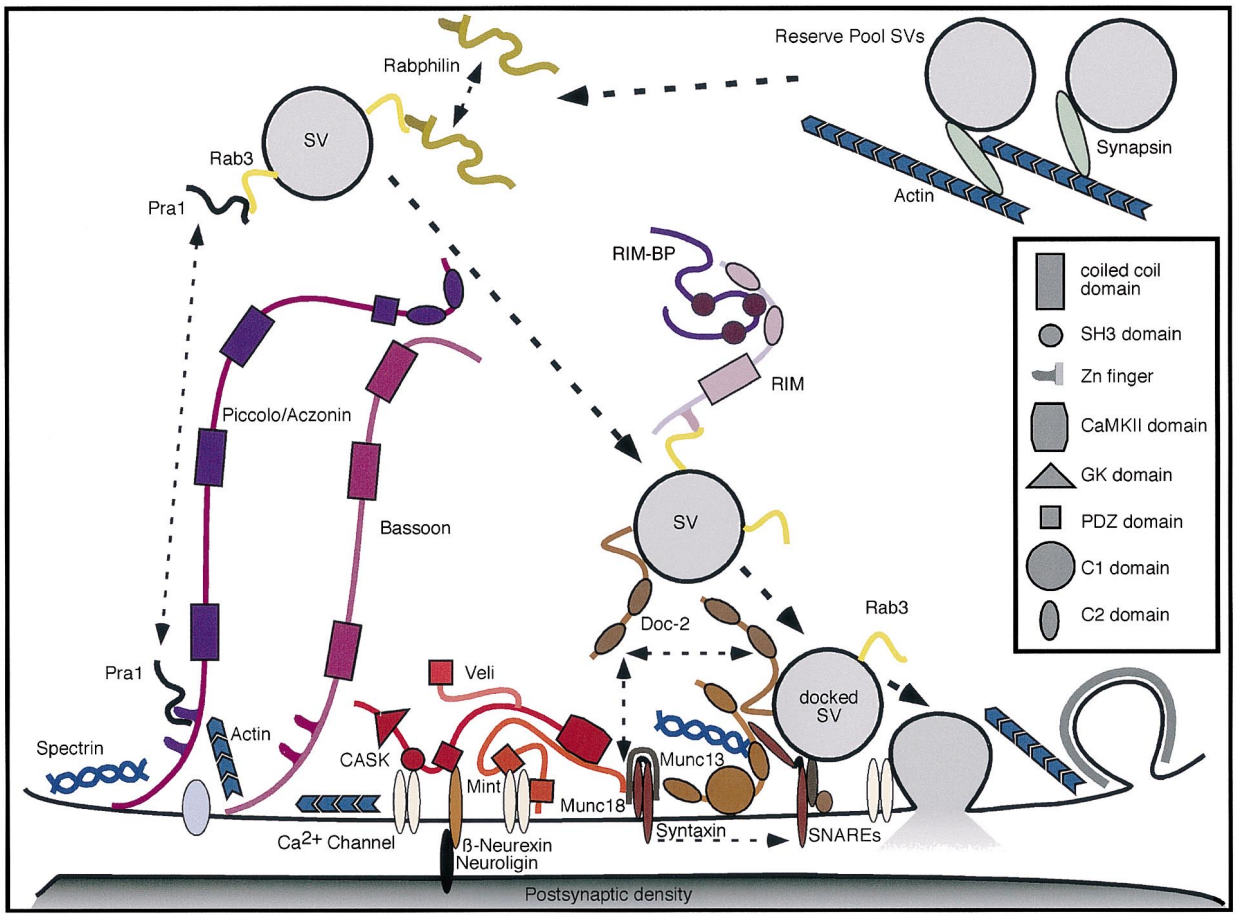

Figure 4. Functional organization of the presynaptic cytomatrix. The model illustrates molecular interactions potentially taking place at the site of neurotransmitter release (for details, see the section on the role of the presynaptic cytomatrix in the functional organization of the neurotransmitter release apparatus).

MAGuKs

MAGuK family members are composed of one or more PDZ domains, a SH3 domain and a guanylate kinase (GuK)-like domain [61, 62]. While SAP90/PSD-95 and SAP97 contain three PDZ domains in the N-terminal half, CASK/LIN2 has a Ca^{2+}/calmodulin-dependent protein kinase (CaMK) domain in front of its single PDZ domain. Generally MAGuKs are involved in the assembly and organization of a variety of cell junctions, including excitatory brain synapses [61–63].

At the presynapse, CASK has been reported to interact with cell adhesion molecules β-neurexin [45] and syndecan 2 [46], presynaptic voltage-gated Ca^{2+} channels [64], as well as the cytosolic proteins Veli/LIN-7 and the Munc18/nSec1-interacting molecule Mint1 [65]. These interactions may serve several functions (fig. 4). On the one hand, β-neurexin binds the postsynaptic cell adhesion protein neuroligin, which in turn is linked to the postsynaptic cytomatrix by the MAGuK SAP90/PSD-95 [66–68], suggesting that this complex is involved in the alignment and/or physical attachment of pre- and postsynaptic membranes. On the other hand, the trimeric CASK/Veli/Mint1 complex may be involved in coupling voltage-gated Ca^{2+} channels to the SV fusion apparatus (see below).

CAZ proteins at retinal ribbon synapses

Ribbon synapses in the retina are designed for continuous release of glutamate from the presynaptic cells, i.e. photoreceptor cells or bipolar cells. The synaptic ribbon is thought to constitute a specific adaptation of the CAZ to this specialized function. The ribbon extends from the presynaptic plasma membrane several hundred nanometers up to 1 µm into the presynaptic terminal. SVs are tethered to the ribbon and steadily transported

to the release site. RIM1 is distributed evenly over the surface of rod photoreceptor ribbon synapses [36]. This distribution is consistent with a role of RIM1 in Rab3-GTP-dependent tethering of SVs to the CAZ.

Bassoon is present at photoreceptor ribbons, but absent from ribbons in bipolar cells [54]. In contrast, Piccolo appears to be present at both types of ribbon synapses [O. Dick and J. H. Brandstaetter, unpublished observation]. Utilizing Ab7f antibodies for immunogold EM revealed that as in conventional synapses the corresponding epitopes are localized about one SV diameter remote from the release site (fig. 3). This supports the view that Bassoon has a geometrically highly ordered arrangement within the CAZ.

Role of the presynaptic cytomatrix in the functional organization of the neurotransmitter release apparatus

Tight spatial and temporal control of organelle-based and molecular events is a prominent feature of synaptic neurotransmitter release. At the level of organelles, this includes formation of vesicle clusters in the vicinity of active zones, movement of SVs within clusters exclusively towards the active zone, as well as restriction of docking and exocytotic fusion to the active zone plasma membrane. At the molecular level, a complex network of protein-protein and protein-lipid interactions allows submillisecond time ranges for Ca^{2+}-triggered exocytosis from docked vesicles in response to arriving action potentials. In this section we will review the involvement of the cytomatrix in organizing such events by surveying three categories of proteins, i.e. classical cytoskeletal proteins such as actin and myosin, the more recently discovered CAZ-specific proteins Munc13, RIM, Piccolo/Aczonin and Bassoon, and several proteins that may be recruited to synapses by interacting with CAZ-specific proteins (see fig. 4).

The actin-based cytoskeleton

Since the detection of actin and myosin in isolated nerve terminals (synaptosomes), the actin-based cytoskeleton has been implicated in neurotransmitter release in a variety of experimental systems (reviewed in [69]). For example, in neuroendocrine cells, actin seems to control the translocation of secretory granules to the plasma membrane in preparation for exocytosis [70, 71]. In synaptosomes, cycles of actin filament assembly and disassembly are involved in transmitter release from large dense core vesicles [72]. Moreover, perturbation of GTP hydrolyzing proteins (GTPases) known to regulate the actin cytoskeleton affected transmitter release in *Aplysia* neurons [73]. Recently it was shown that perturbing actin filament assembly affects synaptic plasticity in the hippocampus, and that this effect includes a change in transmitter release [74]. Mechanistically, actin has been implicated in synaptic transmitter release through its interaction with synapsins [75, 76], a family of peripheral SV membrane proteins that are firmly established as regulators of neurotransmitter release (reviewed in [1, 27, 77]). Whereas synapsins seem to affect exocytosis directly [78, 79], they are also essential for sustaining neurotransmitter release under high-frequency stimulation [80], most likely by maintaining a reserve pool of SVs [25, 79, 81]. Synapsins are thought to keep reserve pool vesicles clustered close to active zones by cross-linking them and tethering them to the actin cytoskeleton [6, 12, 13, 25, 26, 82]. In terms of organizing the presynaptic area, synapsins and actin may mutually influence one another. What argues for an organizing role of synapsins is that synapsins induce actin filament formation and bundling, thus potentially allowing SVs to build their own restricting cage [83, 84]. Conversely, once formed, the actin meshwork is thought to control vesicle mobility by restricting or permitting SV movement, depending on the functional state of the nerve terminal. In keeping with this notion, nonphosphorylated synapsin, which binds both SVs and actin, reduces transmitter release [85], whereas phosphorylation, which dissociates synapsins from vesicles and inhibits their interactions with actin [75, 86], coincides with increased availability of SVs for transmitter release [77]. Because synapsins are phosphorylated during nerve terminal activity [87], they are thought to control synaptic transmission by reversibly anchoring SVs to the cytoskeleton.

Recently another peripheral SV protein, rabphilin3A, has been implicated in controlling actin dynamics by stimulating the actin filament bundling activity of α-actinin [88]. However, this stimulation was inhibited when rabphilin3A was interacting with GTP-rab3A, a monomeric GTPase, which recruits rabphilin3A to SVs [89, 90]. Because Rab3A dissociates from SV during nerve terminal stimulation [91], it has to be assumed that, unlike synapsin, rabphilin3A will not stimulate actin filament bundling while bound to SVs, but only upon detachment from SVs during transmitter release. Hence, the site of rabphilin3A-induced actin filament bundling does not necessarily have to be the CAZ.

Whereas actin-synapsin interactions seem to be involved in restricting SV mobility, actin-myosin interactions have been implicated in mobilizing reserve pool SVs for sustained transmitter release. Using the dye FM1-43 to follow SV exo- and endocytosis, it was shown that inhibitors of myosin light-chain kinase (MLCK), which initiates binding of myosin to actin upon activation by Ca^{2+}, selectively inhibit availability of reserve pool SV for transmitter release, leaving both exocytosis from the readily releasable pool and vesicle

retrieval by endocytosis intact [92]. This finding is supported by the original observation that MLCK inhibitors and antibodies directed against myosin II inhibit synaptic transmission upon microinjection [93, 94]. Later it was shown that another myosin isoform, myosin Va, is a SV-associated protein bound to a binary complex of the SV transmembrane proteins synaptophysin and synaptobrevin [95]. Further studies revealed that myosin Va can catalyze actin-based transport of organelles [96] and that this myosin is necessary for local vesicle movements rather than long-range transport [97]. It may thus be an actin-myosin-based motor system that underlies the exclusively anterograde movement of SVs within clusters as visualized in photobleaching experiments [98]. Interestingly, the work of Prekeris and Terrian also suggested that the completion of an actin-myosin duty cycle is followed by disassembly of the ternary complex of myosin Va, synaptophysin and synaptobrevin [95]. Because binding to synaptophysin is thought to prevent synaptobrevin from performing subsequent actions in exocytosis [99], disassembly of the ternary complex might both finish SV translocation and promote downstream events centered around docking and fusion of SVs.

Role of CAZ proteins in regulated SV exocytosis

The four CAZ-specific proteins known to date are concentrated in the region proximal to the active zone plasma membrane, suggesting functions related to docking and fusion of SV [24]. The functionally best studied CAZ proteins are members of the UNC-13 protein family [48], including *C. elegans* unc-13 [47], rat Munc13 isoforms 1–3 [34, 100] and *Drosophila* Dunc-13 [101]. These proteins share remarkable functional conservation, and studies performed on the three model systems have converged onto the following notion: all family members contain a lipid-binding C1 motif, and both unc-13 and Munc13 have been shown to bind the lipid DAG as well as phorbolesters [35, 102, 103], DAG analogs that stimulate neurotransmitter release [104, 105]. In addition, Munc13-1, Dunc-13 and UNC-13 are essential for both evoked and spontaneous neurotransmitter release at least in a subset of synapses, but not for endocytic retrieval nor for docking of SVs at the active zone plasma membrane ([101, 106, 107], reviewed in [48, 108]). This leaves as possible functions roles in making docked vesicles fusion competent or in exocytotic membrane fusion itself. The fact that α-latrotoxin, a component of black widow spider venom, can cause exocytosis from SVs in Munc13-1-deficient mice, suggested that Munc13-1 primarily acts to confer fusion competence to SVs [107]. While all studies indicated that these proteins are not required for active zone assembly, several observations raise the possibility that Munc13-1 may have a role in recruiting other proteins to the plasma membrane. First, Munc13-1 binds msec7, a molecule that exchanges GDP for GTP on monomeric GTPases and enhances transmitter release [109, 110]. Because Munc13-1 is selectively localized to synapses, it might serve to anchor the cytosolic msec7 at synapses, thus recruiting its release promoting function to the active zone. Second, phorbol esters promote Munc13-1 binding to SV-associated proteins Doc2α and Doc2β [111, 112]. Inhibiting these interactions using competing peptides or fusion proteins reduces transmitter release from neuroendocrine cells [111] and sympathetic neurons [113], and attenuates phorbolester potentiation of release from a CNS synapse, the calyx of Held [114]. Conversely, neurotransmitter release is enhanced by overexpression of Munc13-1 [35]. Moreover, heterologously expressed Munc13-1 translocates to the plasma membrane in response to phorbol ester application and cotranslocates Doc2β when coexpressed in the same cells [35, 112]. Together, these data implicate Munc13-1-Doc2 interactions in neurotransmitter release and raise the possibility that Munc13-1 shuttles between plasma membrane and CAZ as a function of DAG concentrations. Alternatively, as Munc13-1 binds to syntaxin, a plasma membrane protein essential for neurotransmitter release [115], DAG might merely serve to activate Munc13-1 already attached to the membrane for Doc2 binding. Munc13-1 has also been shown to bind a brain-specific isoform of the actin-interacting protein spectrin [116]. Thus, in interacting with proteins of the plasma membrane, SVs, the cytosol and the cortical cytoskeleton as well as with lipids, Munc13 proteins may occupy a central controlling position at the active zone.

RIM1 is less clearly defined in functional terms, but some of its properties point to possible functions. First, heterologous expression of truncated RIM1 affects exocytosis from neuroendocrine cells [36]. Second, its interaction with the SV protein Rab3A provides a link to SVs, and it has been proposed that RIM may anchor SVs at the active zone plasma membrane [51]. Third, unlike Munc13-1 [34], RIM1 cannot be extracted from brain tissue by detergent, suggesting a tight association with the cytomatrix [51]. Together with its multi-domain structure and the recent discovery of further RIM-binding proteins [51], these data suggest that RIMs could act as a scaffolding protein at the active zone plasma membrane.

Scaffolding functions have also been proposed for Bassoon and Piccolo/Aczonin based on their predicted multi-domain structure, exquisite localization at the active zone and intimate association with the CAZ [38–40]. However, to date their actual functions are unknown. Hints to possible roles of Piccolo/Aczonin stem from the identification of interaction partners. The

in vitro binding of Piccolo to PRA1 suggests a function for Piccolo in the regulation of the SV cycle [38]. In addition to Rab3a and VAMP2 [60], PRA1 weakly binds GDP dissociation inhibitor (GDI), a protein involved in the solubilization of membrane-bound Rab GTPases, and appears to regulate the interaction of Rab3A with the SV membrane [117]. The competitive effect of Piccolo-PRA1 interaction on PRA1-Rab3A binding would fit into a hypothetical scenario, in which PRA1 may be displaced from Rab3A as SVs enter the CAZ (fig. 4), and this may influence Rab3A interactions with its effectors, such as the RIMs. Thus Piccolo may be involved in a complex regulatory network that tightly controls the actions of Rab3, which is a key player in Ca^{2+}-triggered SV exocytosis [2]. Binding to profilin implicates Piccolo/Aczonin in dynamics of the actin cytoskeleton [39]. While it is unclear whether or not actin is present in the CAZ, it is well conceivable that, due to their large size, Piccolo/Aczonin as well as Bassoon reach out into the distal pool of SVs. This raises the intriguing possibility that the high molecular weight proteins of the CAZ may coordinate events occurring both in the proximal SV pool and at the transition zone between reserve and proximal pools.

CAZ-associated proteins in active zone assembly and function

The current concept of CAZ function includes the idea that a core of matrix proteins recruits a set of otherwise broadly distributed proteins to synapses. Recent studies have provided the framework for a putative network of CAZ-associated proteins. Of these, CASK/mLin-2 and Mint1/mLin10 have been proposed as candidates for clustering voltage-gated Ca^{2+} channels in the active zone based on their binding to the pore forming α1B subunit of N-type Ca^{2+} channels and their multi-modular structure [64]. In addition, CASK/mLin2 and Mint1/mLin10 (also called X11α) are engaged in a trimeric complex that also includes veli/mLin7 [65, 118, 119]. This complex is conserved between mammals and nematodes, where it is involved in epithelial protein targeting [120], but its function in the mammalian brain is unknown. Whereas the interaction of Cask/mLin2 with neurexins provides a putative link across the synaptic cleft (see above), Mint1/mLin10 offers two possible links between the plasma membrane and SVs. First, Mint1 and the related Mint2 contain a phosphotyrosine binding (PTB) domain, which has been shown to bind the lipid phosphatidyl inositol bisphosphate (PIP2; [121]). Because PIP2 is transiently produced during neurotransmitter release from neuroendocrine cells, and PIP2-generating enzymes reside on secretory vesicles [122, 123], it has been proposed that PIP2 produced on SVs might provide a signal for Mint1 binding [121]. The second link to SVs may be provided by Mint/mLin10 binding to Munc18 [119, 121], which in turn interacts with Doc2 proteins of SVs [124]. Munc18, also called nsec1, rbsec1 and msec1 [125–128] plays a key role in SV exocytosis as evident from the absence of both evoked and spontaneous transmitter release in neurons of Munc18-deficient mice [129]. In addition, Munc18 interacts with the plasma membrane protein syntaxin [130–132], a key component of the exocytotic fusion machinery and major nodal point in the network of presynaptic interactions (reviewed in [133, 134]). Thus, a molecular scaffold comprising CASK/mLin2, Mint1/mLin10, Doc2 and Munc18, all nontransmembrane proteins, could potentially couple SVs to Ca^{2+} channels. However, such coupling can also be accounted for by direct interactions, e.g via binding of synaptotagmin to the synaptic protein interaction (synprint) sites in N-type and P/Q-type voltage-gated Ca^{2+} channels, or via interaction of synaptobrevin with syntaxin and SNAP-25, which also bind the synprint site (reviewed in [135, 136]). Therefore, CASK/mLin2, Mint1/mLin10, Doc2 and Munc18 may rather be involved in tethering SVs to components of the plasma membrane before they are close enough to the active zone plasma membrane to dock by direct interactions. Alternatively, these proteins might be part of a protein machinery that confers fusion competence to docked SVs. Exocytotic fusion itself is thought to be mediated by a docking/fusion core complex consisting of synaptobrevin, syntaxin and SNAP-25 [137–139]. Formation of this complex has been proposed to be regulated by several molecules, including Munc18-1 [128, 140–144], complexins [145, 146], syntaphilin [147] and tomosyn, which dissociates Munc18-1 from syntaxin [148, 149]. Tomosyn is a homolog of the *Drosophila* tumor suppressor protein L(2)gl, which is part of the cortical cytoskeleton and interacts with nonmucle myosin II heavy chain (for references see [148]). Thus, tomosyn may potentially represent a novel link to the cytoskeleton at synapses. Another potential link to the cytoskeleton is provided by the interaction of Munc18 with cyclin-dependent kinase 5 (Cdk5). Both Cdk5, a small kinase sharing sequence homology with mitotically active Cdks, and its activator p35 are essential for correct cortical lamination, suggesting roles in neuronal outgrowth or migration [150, 151]. A Cdk5/p35-complex has been implicated in regulating actin dynamics in neurons via Rac, a GTPase controlling actin polymerization [152]. In addition, p39, another activator of Cdk5, is associated with the actin cytoskeleton [153]. Moreover, Munc18 has been identified as a specific binding partner and stimulator of Cdk5 [154]. Cdk5-mediated phosphorylation of Munc18 dissociates Munc18-syntaxin binding and affects neurotransmitter release from chromaffin cells [155, 156].

In summary, a scenario is beginning to emerge, in which synaptic cell contact, channel clustering and SV exocytosis at active zones can be integrated via a network of molecular interactions. Whereas most of the proteins described in this section are enriched at active zones, none is active zone-specific, and mechanisms governing their recruitment still await discovery. Intriguingly, the interactions of Doc2 and syntaxin with Munc13, as well as of PRA1 and Rab3A with Piccolo/Aczonin and RIM, respectively, provide links for SVs and the plasma membrane, and potentially the entire network of interactions, to the CAZ.

The endocytic machinery

The rapidity and precision of synaptic transmission is critically dependent on the effectiveness of the SV recycling process in the presynaptic nerve terminal. After neurotransmitter release the SV pool needs to be regenerated by endocytic retrieval of SV membranes, which are then refilled with neurotransmitter and retargeted to the active zone. In general, dynamic macromolecular complexes appear to be predestinated to catalyze and regulate the different steps in the recycling process with high speed and efficiency. A meshwork composed of adapter or scaffolding molecules with multiple protein-protein interaction motifs seems to play a pivotal role in the structural organization and/or functional coordination of endocytosis in presynaptic nerve terminals. Furthermore, cortical cytoskeletal structures may be involved at different stages of the endocytic process, membrane invagination, coated pit formation, coated pit sequestration and finally detachment of the newly formed vesicle and movement of this new endocytic compartment away from the plasma membrane into the cytosol (fig. 5). A functional connection between the actin cytoskeleton and endocytic processes, well established in yeast [157], is suggested to also exist in mammalian cells. However, molecular details of this connection are still to be further elucidated (reviewed in [271]). The functional importance of actin polymerization and dynamics in the nervous system has so far mainly been addressed in conjunction with neuronal development. Actin filament dynamics has been shown to be essential for the establishment and movement of growth cones and neurites. Furthermore, Bradke and Dotti [158] have recently demonstrated that local instability of the actin network is essential to establish polarity in neurons. In fully mature synapses, actin filaments localize to both pre- and postsynaptic elements, and recent studies indicate that F-actin disassembles reversibly during SV recycling [159].

In this review we will focus on clathrin-mediated endocytosis, although alternative mechanisms, such as 'kiss and run', may contribute to SV formation at presynaptic nerve terminals (discussed in [5, 9, 160]). Proteins implicated in clathrin-mediated endocytosis are discussed with respect to their physical or functional connection to the actin cytoskeleton and with respect to their involvement in the formation and maintenance of multi-molecular protein networks required for endocytic processes.

The clathrin coat

The formation of clathrin-coated endocytic vesicles at the plasma membrane involves two subsets of proteins, those that are structural components of the clathrin coat and in addition a growing array of accessory proteins (fig. 5). In neurons, both coat components and regulatory molecules are expressed at 10–50-fold higher concentration compared with nonneuronal cells [161], and neuron-specific isoforms or splice variants have been identified for a variety of these proteins (see below).

Endocytic coated vesicle formation is initiated by recruitment of the tetrameric adapter complex AP2 to the plasma membrane [162] and thought to involve AP2 interactions with the SV protein synaptotagmin [163], tyrosine- and dileucine-based sorting signals in the cytoplasmic tails of cargo molecules (reviewed in [164]) and phosphoinositides [165]. Subsequently, the AP2 complexes nucleate the assembly of clathrin triskelia into polyhedral lattices or cages (reviewed in [162]). In addition, the monomeric adaptor protein AP180 binds to both clathrin and AP2 [166, 167]. Whereas the α-subunit of AP2 (α-adaptin) is essential for SV endocytosis [168], AP180 mutants in *Drosophila* and *C. elegans* revealed that the monomeric adaptor protein AP180 regulates SV size [169, 170]. The coassembly of clathrin and adaptor proteins into large heterooligomeric protein complexes is thought to be involved in both forming the nascent transport vesicle and in selectively packing its cargo.

No direct links of clathrin and adaptor proteins to cytoskeletal structures have been identified so far. Recently, HIP1R (Huntingtin interacting protein 1-related; [171]) has been shown both biochemically and by immunolocalization to be stably associated with clathrin-coated pits and vesicles [172]. Whereas the N-terminal part of the protein mediates the association with clathrin-coated pits, the talin-like C-terminal domain of HIP1R binds to F-actin in vitro and colocalizes with cortical F-actin structures in vivo [172]. The ubiquitously expressed HIP1R might thus represent a physical link between clathrin-coated pits and vesicles (or the coat proteins itself) and filamentous actin. In elegant analyses with green fluorescent protein (GFP)-tagged clathrin, coated pits have been demonstrated to reoccur

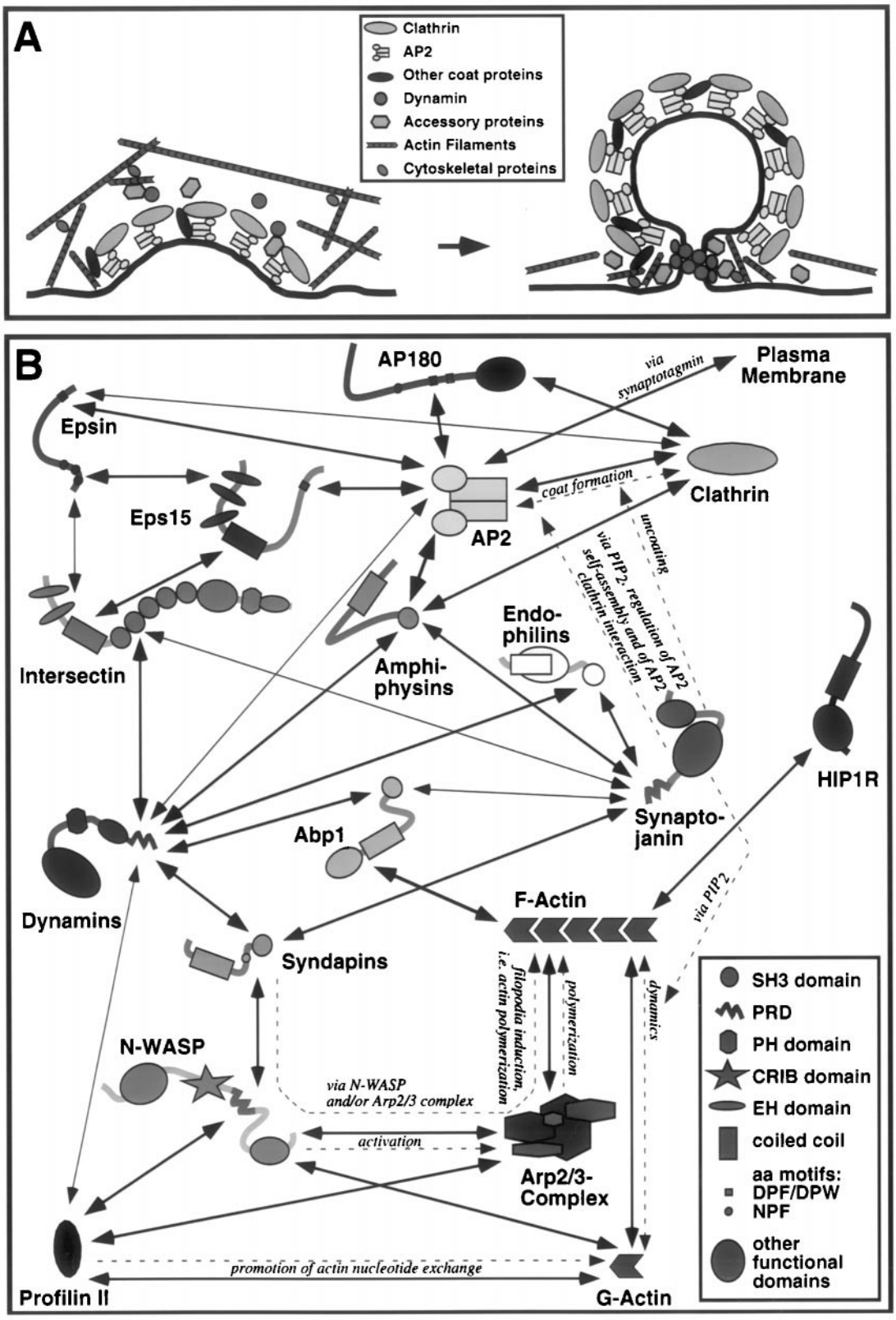

Figure 5. Current view of SV endocytosis. (*A*) Schematic representation of an early step of clathrin-coated pit formation and of a late step, i.e. fission of a constricted clathrin-coated pit leading to an endocytic vesicle. (*B*) The endocytic machinery consists of a complex network of coat proteins, the GTPase dynamin and accessory proteins, several of which can interact with the actin cytoskeleton underlying the plasma membrane (for details, see the section on the endocytic machinery).

at defined sites over time [173]. This implies that coated pit formation is initiated at specific and restricted sites. Cytoskeletal structures may constrain or organize the endocytic machinery at the plasma membrane. Alternatively, the localization of the endocytic machinery to specific sites might be achieved by direct molecular links to the actin cytoskeleton.

In in vitro systems, clathrin and APs alone can form cages on liposomes and are thus sufficient to drive membrane deformation [174]. The formation of a constricted coated pit and the budding and detachment of the vesicle, however, requires a variety of additional factors.

Accessory proteins: the dynamins

The large GTPase dynamin was first implicated in endocytic function when the *Drosophila shibire* mutant that could not recycle SV membranes was shown to be defective in a GTPase, dynamin [175–177]. Work of different groups demonstrated that a similar mutation in the mammalian ortholog, dynamin, results in a block of clathrin-mediated endocytosis [178, 179] and that the endocytic process arrests at the stage of coated membrane invagination [180]. The ability of dynamin to self-assemble into helical structures, either spontaneously [181] or around synaptosomal membranes or lipid vesicles forming membrane tubules [174, 182] and the fact that addition of GTP to dynamin-decorated tubules caused fragmentation [182] led to the view that dynamin may actually be pinching off vesicles, i.e. act as a GTP-driven 'pinchase'. However, some doubt concerning this model raised early on [183] was recently elegantly verified by experiments conducted in the Schmid lab. Overexpression of dynamin mutants defective in the assembly-stimulated GTPase activity did not decrease but instead increased the rate of receptor-mediated endocytosis [184], suggesting that dynamin rather acts as regulatory GTPase which promotes endocytosis in its GTP form [185].

Dynamin was also linked to the actin cytoskeleton. Overexpression of the dynamin K44A mutant was shown to block receptor-mediated endocytosis but also to alter cell shape and redistribute actin stress fibers, an effect which can, however, also be observed during an endocytosis block induced by K^+ depletion or cytosolic acidification [180, 186]. Furthermore, treatment of primary hippocampal cultures with antisense nucleotides to dynamin 1 inhibited neurite outgrowth [187]. In both cases, the defects in cell morphology and cytoskeletal organization might be secondary effects of an endocytosis block.

Besides dynamin itself, which comprises multiple functional domains, including a GTPase domain, a phospholipid-interacting pleckstrin homology domain, a GTPase effector domain, multiple self-assembly sites and a proline-rich domain (PRD) (reviewed in [185]), and is one of the key players within the vesicle formation process, several dynamin-interacting proteins display functional links to the actin cytoskeleton. Thus far all of these proteins seem to interact with the C-terminal PRD of dynamin via polyproline binding or SH3 domain interactions.

Dynamin-interacting proteins involved in endocytosis

A variety of these SH3 domain-containing proteins have been implicated in clathrin-mediated endocytosis [188–193], such as amphiphysins 1 and 2 [194–197], endophilin [198, 199], DAP160/intersectin [200, 201] and syndapin I and II [193, 202]. These PRD/SH3 domain interactions are essential to dynamin function. SH3 domains stimulate the GTPase activity of dynamin 1 in vitro [203, 204]. Additionally, recruitment of dynamin to clathrin-coated pits is critically dependent on the amino acid sequence of a potential SH3 domain recognition site within its PRD [205], suggesting the involvement of SH3 domain-containing proteins in dynamin targeting.

PRD/SH3 domain interactions seem to be crucial for SV recycling. When injected into the presynaptic compartment of lamprey neurons, the SH3 domain of amphiphysin or a dynamin peptide containing the SH3 binding site impaired SV recycling and caused the accumulation of invaginated clathrin-coated pits [189]. A similar dynamin peptide inhibited SV endocytosis in synaptosomes [206]. In an in vitro PC12 cell system, the PRD of dynamin impaired the formation of SVs [207]. In line with this, immunoprecipitation studies have demonstrated that PRD- and SH3-domain-containing proteins form large, multimeric and stable protein complexes in the nerve terminal [194, 197, 198, 202]. An appealing conjecture is that these molecules are linked together via their PRDs and their SH3 domains into protein machines that help form SVs at endocytosis 'hot spots' [168, 200, 208] at the nerve terminal plasma membrane.

Endophilins

Endophilin's role in SV recycling has been demonstrated in two recent studies. First, microinjection of antibodies to endophilin into the lamprey giant axonal synapse caused accumulation of shallow coated pits and a depletion of SVs in tonically stimulated synapses [209]. Second, depletion of endophilin from cytosol interfered with synaptic-like microvesicle formation in perforated PC12 cells [210]. These authors also characterized endophilin 1 as a lysophosphatidic acid acyl transferase and proposed a model in which endophilin,

by converting lysophosphatidic acid to phosphatidic acid, induces negative membrane curvature at the edges of clathrin-coated pits and thus facilitates endocytosis.

Intersectins

Proteins of the intersectin/DAP160 family are suggested to function as scaffolding molecules organizing SH3 and Eps15 homology (EH) domain-binding proteins at endocytic sites [200]. They interact with dynamin via their SH3 domains [200], with Eps15 [191] and with intersectin binding protein 2/epsin via their EH domains [191, 211] and might in addition link the endocytic machinery to exocytosis because they bind to SNAP-25 via their middle domain [212]. Both epsins, which are enriched in presynaptic nerve terminals [213], and Eps15 are linked to the clathrin coat; they bind to the appendage domain of α-adaptin via DPW/DPF motifs. Overexpression of the AP2-binding part of either protein interferes with receptor-mediated endocytosis (reviewed in [214]). Additional ligands for the appendage domain of α-adaptin include amphiphysin 1 and 2, auxillin, AP180 and the 170-kD isoform of synaptojanin ([167] and references therein; [215]), indicating multiple protein-protein interactions between coat components and accessory factors of the endocytic machinery.

Amphiphysins

An extensively studied family of dynamin-interacting proteins consists of amphiphysins (reviewed in [216]). Amphiphysin 1 was shown to interact with dynamin [194], AP2 [194, 217] and clathrin [196, 218], suggesting that they may target dynamin to the clathrin lattice [205]. The SH3 domains of amphiphysin 1 and 2 prevent dynamin ring formation in solution [190], suggesting that amphiphysin would have to dissociate in order to allow dynamin self-assembly. On the other side, Takei et al. [219] showed that amphiphysin is able to transform spherical liposomes into narrow tubules and enhances the liposome-fragmenting activity of dynamin 1 in the presence of GTP, an effect independent of dynamin recruitment. Amphiphysin function was also linked to the actin cytoskeleton by antisense experiments. Treatment of primary hippocampal cultures with antisense nucleotides to amphiphysin 1 inhibited neurite outgrowth and caused the collapse of growth cones [220]. This result is rather unlikely to be a secondary effect, at least not of membrane-trafficking perturbations, since the treatment affected neither receptor-mediated nor fluid-phase endocytosis. The link to the actin cytoskeleton is likely to involve further proteins, because no direct interaction of amphiphysins with cytoskeletal components has been unraveled so far.

Syndapins

Recently, a second family of dynamin-interacting proteins with functional connections to the actin cytoskeleton has been established, the SH3 domain-containing syndapins [193, 202]. Genes of mouse orthologs of syndapins were also described and termed PACSINs [221]. Thus far, the family contains two isoforms, the brain-specific syndapin I and the more ubiquitously expressed syndapin II, which exists in four splice variants. The isoforms show a high degree of similarity, and both interact with a subset of proteins involved in membrane trafficking: dynamins, synaptojanins and synapsins [193, 202].

Additionally, syndapins interact with N-WASP, the more ubiquitously expressed, brain-enriched isoform of the Wiskott Aldrich syndrome protein [222], a potent activator of the Arp2/3 complex actin-polymerization machinery [223–226]. WASP proteins interact with the Arp2/3 complex through their very C-terminus [227]. They also bind to actin and Rho-type GTPases. N-WASP is specifically activated by Cdc42-GTP and induced extremely long actin microspikes upon coexpression with a dominant-active Cdc42 mutant [228]. The syndapin interaction and the fact that lymphocytes from WASP knockout mice exhibited both a reduction in actin polymerization and defects in T-cell-receptor endocytosis [229] suggest that mammalian WASPs might additionally exhibit endocytic functions. Both functional aspects of syndapins, their role in endocytosis and their role as mediators of the actin cytoskeleton have been examined in vivo. Dominant-negative experiments demonstrated that the SH3 domain of both syndapin isoforms inhibited receptor-mediated internalization of transferrin [193]. This block occurs late in the endocytic vesicle formation process, in the scission event, and requires a membrane-associated factor, as revealed in an in vitro reconstitution system [192]. These results are consistent with the fact that syndapins interact with dynamin. Overexpression of full-length syndapins, but not of the N-terminal part or the SH3 domains alone, had a strong effect on cortical actin organization and induced filopodia. This syndapin overexpression phenotype appears to be mediated by the Arp2/3 complex at the cell periphery because it was completely suppressed by coexpression of a cytosolic C-terminal fragment of N-WASP, which perturbs the recruitment of the Arp2/3 complex to the plasma membrane and activates it within the cytosol [193]. Since the SH3 domain alone was sufficient to block endocytosis but did not induce filopodia, it was concluded that the syndapin-induced cytoskeletal rearrangements are not an indirect consequence of the inhibition of receptor-mediated endocytosis but require activation of the Arp2/3 actin polymerization machinery. Consistent with a role in actin dynamics, syndapins

localized to sites of high actin turnover such as filopodia tips and lamellipodia. A splice variant of a chicken ortholog of syndapin, FAP52, was described as a component of focal adhesions, which are also specialized actin structures with high dynamics [230]. These results, however, could not be repeated for a mouse ortholog [221].

A variety of steps of the endocytic cycle may exhibit links to the actin cytoskeleton via syndapins. Syndapins could couple dynamin GTPase activation to actin polymerization and act as a key player in the ignition and timing of an actin polymerization burst. This polymerization burst may be involved in force generation to facilitate the fission event at the neck of clathrin-coated pits. Furthermore, it may help to detach the newly formed vesicle from the membrane and to propel newly formed endocytic vesicles through a viscous cytoplasm away from the plasma membrane. Both pinosomes and clathrin-coated vesicles have recently been described associated with actin comet tails in the cytoplasm [231, 232], so have endosomes and lysosomes, which exhibited N-WASP staining at the interface of the actin tail and the vesicle, in in vitro systems [233]. A polarity of N-WASP recruitment and actin polymerization during the endocytic process and thus a directed propulsion could be achieved by recruitment of the actin polymerization machinery to the neck of a coated pit via dynamin and syndapin. In order to fulfill such a role, however, syndapins would have to interact with dynamin and N-WASP simultaneously. Syndapins comprise multiple protein interaction interfaces that include potential oligomerization domains; thus, simultaneous interactions may well be possible, or additional proteins may be involved.

A potential increase of actin dynamics mediated by N-WASP and Arp2/3 at sites of endocytosis would also be consistent with the hypothesis that promotion of actin treadmilling may be required for endocytic processes in order to remove the physical barrier of the cortical cytoskeleton.

The actin-binding proteins profilin and Abp1

Dynamin furthermore interacts with two proteins that directly associate with G-actin and F-actin, respectively, profilin and Abp1. Profilins are small G-actin-binding proteins that catalyze nucleotide exchange on the slow ATPase actin [234]. The role of profilins in the regulation of actin dynamics [235] might be additionally mediated via their interaction with modulators of actin filament polymerization, the Arp2/3 complex [236] and proteins of the VASP/MENA/diaphanous family [237–239]. Via the same polyproline-binding properties, the brain-enriched profilin 2 is able to interact with dynamin and synapsins as shown by affinity chromatography of mouse brain extracts, suggesting a role for profilin 2 in membrane trafficking events in the brain [240]. The addition of recombinant profilins 1 and 2 to diluted cytosol has been reported to stimulate the biogenesis of SV-like microvesicles in a perforated PC12 cell system [241], but it remained unclear whether profilins are a necessary component for the formation of SV-like microvesicles [210]. A potential role of profilins might be to increase the local concentration of G-actin and to promote F-actin assembly at sites of endocytosis.

Another attractive candidate to be a functional and/or physical link between the endocytic machinery and the actin cytoskeleton is the mammalian homologue of yeast Abp1, mAbp1 [242], also identified as SH3P7 in a screen for polyproline-binding sequences [243] and as a Src substrate [244, 245]. Mouse Abp1 binds specifically to F-actin in vitro and in vivo using two different functional domains. The protein is preferentially associated with dynamic actin structures [242], it accumulates in lamellipodial areas of cell growth and in lamellipodial sheets of spreading and moving cells [242], indicating an involvement in actin dynamics. However, to date no effects for bacterially expressed mAbp1 on F-actin polymerization and depolymerization have been detected in vitro [242]. This may require further proteins and/or factors. Several SH3 domain-binding proteins of the mouse ortholog of Abp1 were identified. The interaction of the mAbp1 SH3 domain with dynamin, synapsin 1 and synaptojanin indicates that Abp1 could play a role in membrane trafficking events in mammalian cells. Overexpression of the C-terminal SH3 domain of mouse Abp1 leads to a drastic reduction of the receptor-mediated uptake of transferrin in vivo [M. Kessels et al., unpublished results]. Abp1 may thus be linked to the endocytic machinery via its SH3 domain and to the actin cytoskeleton via its two N-terminal actin-binding modules. Considering these properties, it can be hypothesized that Abp1 plays a role in the organization of the endocytic machinery at the cell cortex and/or coordinates the endocytic and cytoskeletal function in a timely or spatial manner. Since the actin-binding protein interacts with dynamin, which is asymmetrically distributed during the fission reaction of the newly formed vesicle, it could participate in the proposed ignition of actin tail formation.

Synaptojanin

Other possible candidate molecules to act at the interface of endocytosis and actin dynamics are proteins involved in the generation or metabolism of regulatory components such as phosphoinositides, which affect signaling processes, membrane-trafficking events and

the actin cytoskeleton (reviewed in [246, 247]). One such protein is the polyphosphoinositide phosphatase synaptojanin 1 [248], which catabolizes PIP2 and PIP3 [249]. Synaptojanin interacts with several accessory proteins implicated in clathrin-mediated endocytosis via its proline-rich domain, amphiphysin [194], endophilin [199, 250], DAP160/intersectin [200] and syndapin [202]; the 170-kDa splice form associates with Eps15 [251], clathrin and AP2 [215]. In neurons of synaptojanin 1-deficient mice, clathrin-coated vesicles accumulate in nerve endings and in hippocampal slices of these mutant animals, and enhanced synaptic depression during prolonged high-frequency stimulation followed by delayed recovery was observed [252]. These results suggest an involvement for synaptojanin 1 in the uncoating of endocytic vesicles. Considering the pleiotropic roles of phosphoinositides, the cellular function of synaptojanin might not be limited to the regulation of membrane traffic. PIP_2 regulates actin polymerization and depolymerization via binding to a variety of actin-regulatory proteins, and a decrease in the amount of PIP_2 is believed to lead to local actin depolymerization (see references cited in [253]). Synaptojanin has been shown to be able to hydrolyze PIP_2 bound to actin-regulatory proteins such as profilin, cofilin and α-actinin in vitro [253], and overexpression of synaptojanin, but not a phosphatase-negative mutant in COS-7 cells, caused rearrangement of stress fibers [253]. Synaptojanin might thus, in a concerted action, promote both the uncoating of vesicles and local actin turnover. Such properties might be required to remove physical barriers for the inward movement of now uncoated endocytic vesicles.

As outlined above, each step of SV endocytosis, i.e. membrane invagination, coated pit formation, coated pit sequestration, detachment of the newly formed vesicle and movement of this new endocytic compartment away from the plasma membrane into the cytosol, is dependent on the formation of multimeric protein complex and might also involve the actin cytoskeleton. Studies at the *Drosophila* neuromuscular junction indicate that in close vicinity to areas of active exocytosis, similar areas of active compensatory endocytosis exist. These 'hot spots' of endocytosis are selectively enriched in different endocytic proteins such as dynamin, α-adaptin and DAP160 [168, 200, 208]. Thus, the machinery for the recovery of SV membrane and protein constituents appears not to be freely diffusible, but instead concentrated and anchored in close proximity to the active zone. An involvement of the presynaptic cytomatrix and the cortical cytoskeleton in the formation and maintenance of these areas specialized for individual steps in the SV cycle is most likely.

Assembly of the active zone during development

The assembly of a functional active zone is a key event during the genesis of CNS synapses. The formation of synapses presumably begins with the protrusive activity of one or both of the future synaptic partners and the establishment of a new axo-dendritic physical contact. Subsequent steps include the assembly of a specialized junctional cytoskeletal matrix [55, 254, 255] that serves to stabilize the adhesion site as well as to promote the recruitment and clustering of neurotransmitter receptors, ion channels and SVs. Real-time imaging studies both in slices and hippocampal cultures have shown that individual synaptic connections may form fairly quickly [255–264]. For example, activity-induced recycling of SVs has been observed as soon as 30 minutes after the initial axo-dendritic contact [255, 264]. In contrast, the recruitment of glutamate receptors, presumably to the postsynaptic compartment, seems to be delayed on average by another 40 min [255]. These studies suggest that synaptogenesis involves a sequential series of events and that the presynaptic active zone may become functionally active prior to the maturation of postsynaptic reception apparatus. Nonetheless, the postsynaptic side may critically influence the formation of presynaptic transmitter release sites. A recent study by Scheiffele et al. has shown that synaptic cell adhesion molecule neuroligin is able to trigger presynaptic differentiation when expressed in nonneuronal cells [265].

At present the cellular mechanisms that underlie the establishment of the CAZ and the PSD remain unclear. Recent studies examining how and in what form active zonal proteins are transported to nascent synapses are beginning to shed some light on this important issue. Studies by Ahmari et al. have shown that clusters of vesicles containing synaptic proteins are found at sites of newly forming synapses, suggesting that nerve terminals may be assembled in part from preformed complexes [264, 266]. These vesicular clusters potentially represent a collection of precursor vesicles, each involved in the assembly of specific presynaptic subdomains such as active zones and perisynaptic plasma membranes. With regard to the formation of active zones, a 80-nm dense core vesicle has been observed at sites of synapse formation, e.g. in the developing spinal cord in association with small patches of active zonal material apposed to modest PSDs [267]. Morphologically, these dense core vesicles, with electron-dense projection on their surface called spicules, closely resemble the CAZ associated with mature synapses [267], suggesting that these granulated vesicles may represent precursors of the active zonal plasma membrane. Studies by Zhai et al. designed to address how the CAZ proteins Piccolo and Bassoon are transported and recruited to nascent synapse support this concept. Using

cultured hippocampal neurons as a model system of synaptogenesis, they showed that Piccolo and Bassoon are expressed at early stages of neuronal differentiation and arrive at newly forming synapses prior to or simultaneously with the acquisition of evoked SV recycling [55, 255, R. Zhai, H. V. Friedman, C. Cases-Langhoff et al., submitted]. Moreover, both proteins have been found to be transported to nascent synapses on the surface of an 80-nm dense core vesicle together with the synaptic adhesion molecule N-cadherin, as well as the t-SNAREs (target SNAP receptors) syntaxin and SNAP-25 [R. Zhai, H. V. Friedman, C. Cases-Langhoff et al., submitted]. SVs as well as peri-synaptic proteins were found on distinct pools of vesicles. Clearly the fusion of this precursor dense core vesicle with the plasma membrane at nascent synapse would allow for the rapid establishment of new active zones. In this scenario, vesicle-associated adhesion molecules would facilitate the formation of stable trans-synaptic cell-cell adhesion sites and the subsequent recruitment and localization of postsynaptic structural proteins and neurotransmitter receptors [268]. Moreover, the insertion of CAZ proteins such as Piccolo and Bassoon as well as components of the SV exocytotic machinery would expedite the rapid acquisition of activity-induced SV recycling. Finally, assuming that these dense core vesicles contain neuropeptides, neurotrophic factors and extracellular matrix proteins, it is likely that the release of this material into the nascent synaptic cleft could also induce postsynaptic differentiation as shown for agrin and neuregulin at the neuromuscular junctions [269, 270]. As such, the fusion of this active zone precursor vesicle would both expedite the assembly of the CAZ as well as confine subsequent fusion of SVs to this site.

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