Published in final edited form as:
Mol Neurobiol. 2009 April ; 39(2): 130–148. doi:10.1007/s12035-009-8058-z.

The molecular architecture of ribbon presynaptic terminals

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Abstract

The primary receptor neurons of the auditory, vestibular, and visual systems encode a broad range of sensory information by modulating the tonic release of the neurotransmitter glutamate in response to graded changes in membrane potential. The output synapses of these neurons are marked by structures called synaptic ribbons, which tether a pool of releasable synaptic vesicles at the active zone, where glutamate release occurs in response to calcium influx through L-type channels. Ribbons are composed primarily of the protein, RIBEYE, which is unique to ribbon synapses, but cytomatrix proteins that regulate the vesicle cycle in conventional terminals, such as Piccolo and Bassoon, also are found at ribbons. Conventional and ribbon terminals differ, however, in the size, molecular composition, and mobilization of their synaptic vesicle pools. Calcium-binding proteins and plasma-membrane calcium pumps, together with endomembrane pumps and channels, play important roles in calcium handling at ribbon synapses. Taken together, emerging evidence suggests that several molecular and cellular specializations work in concert to support the sustained exocytosis of glutamate that is a hallmark of ribbon synapses. Consistent with its functional importance, abnormalities in a variety of functional aspects of the ribbon presynaptic terminal underlie several forms of auditory neuropathy and retinopathy.

Keywords

sensory; hair cell; retina; pinealocyte; RIBEYE; L-type calcium channel; glutamate; synaptic vesicle; exocytosis; endocytosis

Introduction

The synapses of vertebrate sensory receptor cells transmit a broad range of information with high fidelity over a prolonged period of time. For example, human photoreceptors can release neurotransmitter tonically for hours and can signal changes in light intensity over a dynamic range of 10^{10} [1]. Retinal bipolar cells, which receive inputs from photoreceptors, also propagate signals via graded, sustained changes in neurotransmitter release to their postsynaptic partners—amacrine cells and retinal ganglion cells—in the inner retina. In poikilotherms, pinealocytes resemble retinal photoreceptors and can also relay photic information to targets such as pineal ganglion cells (reviewed in [2]). Like photoreceptors, hair cells of the auditory, vestibular, lateral line, and electroreceptor organs are exquisitely sensitive and continually transmit graded changes in membrane potential [3].

The synaptic terminals of all of these sensory neurons share a specialized organelle, the synaptic ribbon (Fig. 1). Also termed synaptic bodies or dense bodies, ribbons are proteinaceous organelles that tether large numbers of synaptic vesicles near the active zone, where neurotransmitter release occurs. The importance of the ribbon in synaptic transmission was revealed with the discovery of visual [7,8] and auditory [9] deficits in mutants that lack anchored ribbons. Over the past two decades, substantial progress has been made in the characterization of the proteomes of ribbon presynaptic terminals, and investigations of mouse and zebrafish mutants that affect ribbons have provided new insights into their functions. In this review, we describe the molecular and cellular biology of adult ribbon presynaptic

terminals, with an emphasis on the synaptic vesicle cycle and calcium homeostasis in retinal and hair-cell ribbon terminals.

Molecular composition of the synaptic ribbon

Synaptic ribbons were originally identified in electron micrographs as electron-dense, osmiophilic structures surrounded by vesicles in the presynaptic terminals of photoreceptors and hair cells ([10-13]; see Fig. 2). These heterogeneous organelles vary in shape, size, and number of tethered vesicles depending on activity. Enzymatic digestion of ribbons suggested they are proteinaceous [16], but the molecular characterization of the synaptic ribbon did not progress further until the production of the B16 monoclonal antibody, which immunolabels retinal, pineal [17], and hair cell ribbons [18] and binds to proteins of a variety of different sizes in Western blots of retinal homogenates [17]. However, although at least one peptide epitope recognized by B16 has been characterized [19], it is not clear which of the multiple antigens identified in Western blots might be the component of the synaptic ribbon labeled by B16 in immunocytochemistry experiments.

The logjam in molecular characterization of the ribbon was broken by Schmitz and colleagues [20], who used partial purification of retinal ribbons to identify RIBEYE as a specific and major component of the ribbon. RIBEYE contains a serine- and proline-rich amino-terminal A domain and a carboxyl-terminal B domain that is identical to all but the amino-terminal 20 residues of CtBP2, a transcriptional repressor related to D-isomer-specific 2-hydroxyacid dehydrogenases. Consistent with the notion that synaptic ribbons are vertebrate specializations, no RIBEYE orthologs exist in the Drosophila and C. elegans genomes. However, vesicles are associated with ribbon-like structures called T-bars at active zones of many synapses in Drosophila (reviewed in [21]), suggesting that invertebrates possess alternative molecular mechanisms to achieve the synaptic function of ribbons. The molecular composition of T-bar ribbons is not yet known. Besides retinal ribbon synapses, RIBEYE appears to be expressed only in vertebrate pinealocytes [20] and hair cells [22]. Immunoelectron micrographs reveal that RIBEYE localizes to the ribbon [23]. It has been estimated that RIBEYE (possibly in association with CtBP1; see below) constitutes 64-69% of the total volume of a goldfish bipolar cell ribbon [24].

Although a RIBEYE knockout has not yet been reported, zebrafish with decreased levels of a RIBEYE ortholog have an impaired optokinetic response and retinal ribbon abnormalities [25]. It is unclear whether the aberrant ribbons in these morphants result from specific defects in ribbon formation or from secondary effects of abnormal bipolar cell development and increased apoptosis. RIBEYE can polymerize via interactions between its A and B domains to form vesicle-associated structures reminiscent of spherical synaptic ribbons. NAD(H) may promote the assembly of synaptic ribbons by favoring homotypic, and inhibiting heterotypic, interactions between these domains. Additional proteins may be necessary to generate plate-like ribbons from the spheres [26].

Although RIBEYE is still the only known protein specific for the synaptic ribbon, the molecular composition of the ribbon is beginning to be elucidated. CtBP1/BARS, a CtBP2 homolog, clusters at photoreceptor [23] and pinealocyte [27] ribbons. Because CtBP1 and CtBP2 form heterodimers in transcriptional complexes, CtBP1/BARS may be recruited to ribbons by interacting with the B domain of RIBEYE, which is nearly identical to CtBP2. Unlike RIBEYE, CtBP1/BARS is also found at conventional synapses [23]. Besides its role as a transcriptional co-repressor, CtBP1/BARS has been implicated in intracellular membrane trafficking, membrane fission, and regulation of the microtubule cytoskeleton (reviewed in [28]). Ultrastructural evidence suggests that endocytosis occurs lateral to the active zone at ribbon synapses [29], so it is difficult to envision how CtBP1/BARS associated with the ribbon could

be involved in the endocytotic limb, unless it shuttles on and off the ribbon. By affecting membrane curvature, it is possible that CtBP1/BARS could influence exocytosis at the ribbon.

Over the past decade, additional proteins have been localized to the synaptic ribbon. For example, photoreceptor [30] and pinealocyte [27] ribbons express the kinesin isoform KIF3A, which associates with KAP3 and either KIF3B or KIF3C to form the kinesin II holoenzyme [31-33] that mediates anterograde transport along microtubules [33]. Conditional inactivation of KIF3A in photoreceptors results in the ectopic accumulation of opsin and membrane in the inner segment, followed by apoptosis [34,35]. The synaptic terminals of photoreceptors from these mutant mice were not examined, so the function of KIF3A at ribbons is unknown. One possible function could be to transport synaptic vesicles down the ribbon to the active zone, like a conveyor belt [16]. However, the other components of the kinesin II holoenzyme do not appear to be expressed in photoreceptor terminals [30]. Electrostatic interactions prevent efficient KIF3A homodimerization [36], and it is unclear if monomeric KIF3A could support movement. Other kinesin monomers can travel along microtubules, however [37,38]. Because microtubules are not found at ribbons [39], KIF3A would need to walk down the ribbon by interacting with some other component of the ribbon.

Cytomatrix proteins assemble at the synaptic ribbon and its surrounding environment

At least five families of cytomatrix proteins make up the filamentous strand network that may organize synaptic vesicle trafficking at the active zone of conventional terminals. These families include liprins, RIMs, Munc13s, CASTs/ERCs/ELKS, Piccolo and Bassoon. These multidomain proteins interact extensively with each other and have diverse activities that only recently have begun to be elucidated (reviewed in [40,41]). Except for the liprins, members of each cytomatrix protein family have been found to be concentrated at the synaptic ribbon (Fig. 3). RIM1 was the first protein to be identified at synaptic ribbons [42]. Despite this, its role at ribbons is unknown. At conventional synapses, RIM1 interacts with Munc13-1 via an amino-terminal zinc finger to prime synaptic vesicles for fusion [43]. This interaction and function may be conserved since Munc13-1 is present at ribbon synapses [23], and it has been suggested that priming of vesicles associated with the ribbon may occur [44]. ELKS/CAST2/ERC1 and Piccolo may be a part of this complex since they are found on ribbons [23,45] and can interact with RIMs [46,47].

Filamentous strands have long been known to connect the base of synaptic ribbons to an aggregate (called the arciform density by [48]) or aggregates [49] of electron-dense material closely apposed to the plasma membrane. Bassoon, an enormous cytomatrix protein of 420 kD, is found around the base of ribbons [50] and may correspond to the anchoring filaments. Consistent with this localization and function, anchored ribbons are absent from photoreceptors [7] and hair cells [9] in mutant mice that lack the central core of Bassoon. Electroretinograms (ERGs) revealed impaired transmission between photoreceptors and bipolar cells in these mice, which also displayed an auditory neuropathy caused by a deficit in fast, synchronous neurotransmitter release from cochlear inner hair cells. However, sustained neurotransmitter release was unaffected in the absence of attached ribbons in cochlear hair cells. The basis for maintained release at these synapses is unclear, but the result has called into question the notion that ribbons are important for sustained release.

The central core in Bassoon can bind to RIBEYE and CtBP1 [23]. While the molecular links at the arciform density and active zone are unknown, an attractive candidate is CAST1/ERC2, because it directly interacts with Bassoon [51] and is found beneath retinal ribbons [23]. Additional studies are needed to identify the molecular composition of the arciform density and to determine the roles of the other cytomatrix proteins at ribbon terminals.

Calcium influx through L-type channels, clustered below the ribbon, drives rapid and sustained neurotransmitter release

Directly aligned with the arciform density are 60-400 polyhedral, intramembranous particles [52,53] thought to be calcium channels ([54]; see Fig. 4). Influx of calcium occurs through voltage-gated channels at hotspots, presumably corresponding to the calcium channel clusters, that co-localize with ribbons [22,55]. Blocking this calcium current with dihydropyridines in bipolar cells [56], photoreceptors [57], and hair cells [58] decreases neurotransmitter release. Sensitivity to dihydropyridines classifies the calcium current as L-type. This current also exhibits rapid activation at relatively hyperpolarized membrane potentials, rapid deactivation, and very slow inactivation [59-61]. L-type calcium channels cluster at ribbon-type active zones [62-64] in close proximity to synaptic vesicles, allowing for rapid stimulus-secretion coupling [61,65].

Photoreceptors release neurotransmitter continuously at rates between 1-100 vesicles/second/active zone (reviewed in [66]). Calcium channels that inactivate slowly are a prerequisite to sustain such release. The Cav1.4 (α1F) pore-forming subunit exhibits particularly slow inactivation and is mutated in patients with incomplete congenital stationary night blindness (CSNB2) [67,68]. These patients have reduced visual acuity, especially at night, due to abnormal rod and cone function. Morgans [62] localized Cav1.4 to active zones in rod synaptic terminals, and Cav1.4 mouse mutants have reduced b-waves in ERGs [69], consistent with a role for Cav1.4 channels in synaptic transmission between photoreceptors and second-order retinal neurons. 90% of depolarization-induced calcium influx into photoreceptor terminals is lost in Cav1.4 knockouts, confirming the essential role of this subunit in photoreceptors. The Cav1.4 α subunit appears to assemble with the β2 [70] and α2-δ [71] auxiliary subunits, since mutations in these subunits lead to similar retinopathies. These auxiliary subunits help to shape the electrophysiological properties of the L-type channel in photoreceptors (reviewed in [66]).

Although photoreceptors and inner hair cells tonically release neurotransmitter, these two cell types utilize different calcium channels to release neurotransmitter and control electrical tuning. Cav1.3 (α1D), which also forms L-type calcium channels, is robustly expressed in cochlear hair cells [72]. Knockouts display profound deafness as revealed by lack of motor responses to auditory stimuli and an increased threshold for auditory brainstem responses [73,74]. Loss of Cav1.3 abolishes 97% of the calcium current in outer hair cells [75] and 90% in inner hair cells [73], leading to dramatically decreased exocytosis [76]. Vestibular function appears to be normal in the mouse mutant [73,74], but zebrafish mutants lacking a Cav1.3 ortholog have both auditory and vestibular dysfunction [77]. The Cav1.3 mouse mutant also has a normal ERG [78], despite reports of Cav1.3 expression in rods [79], cones [80], and bipolar cells [81].

While Cav1.3 and Cav1.4 constitute the major calcium channels in hair cells and photoreceptors, respectively, their biophysical properties differ somewhat from the native channels at ribbon synapses. Like Cav1.4, Cav1.3 displays little inactivation at ribbon presynaptic terminals [82]. However, Cav1.3 becomes inactivated very quickly by calcium when expressed in heterologous cells [83], suggesting the existence of an inhibitor of calcium-dependent inactivation (CDI) in hair cells. Possible candidates are members of the CaBP family, calmodulin-like calcium-binding proteins that modulate voltage-gated calcium channels. Indeed, CaBP1 and CaBP4 block CDI of Cav1.3 in heterologous cells [84,85]. Since CaBP4 knockout mice are not deaf and display normal calcium influx and exocytosis, CaBP1 may be the dominant regulator of Cav1.3 in inner hair cells [85]. CaBP4 shifts the activation curve of Cav1.4 in the negative direction by 10-15 mV, increasing calcium influx five-fold at the photoreceptor resting potential of -40 mV [86]. Underscoring its importance in photoreceptor synaptic transmission, mutations in CaBP4 lead to CSNB2 [87]. CaBP4

knockout mice phenocopy the genetic deletion of Cav1.4 or its associated subunits [86]. Taken together, these results suggest that specific L-type calcium channels and their modulators are essential for calcium influx and subsequent synaptic vesicle exocytosis at ribbon synapses.

Mechanisms of exocytosis at ribbon presynaptic terminals

Ultrastructural evidence suggests that vesicles fuse at active zones lateral to presumptive calcium channels at synapses where ribbons nestle within an evagination of the plasma membrane ([29]; see Fig. 4). Imaging of vesicles labeled with FM dye recently confirmed that ribbon-associated vesicles undergo exocytosis [88]. It is believed that the vesicles docked at the plasma membrane constitute the readily releasable pool that exocytoses first, depleting with a time constant of 0.5 milliseconds in goldfish MB1 bipolar cells [89]. Capacitance measurements have also identified a slower kinetic component of exocytosis that corresponds, in goldfish MB1 bipolars, to the total number of vesicles attached to ribbons [90]. The morphological correlate for this slower releasable pool is currently unclear at other ribbon synapses and may reflect the exocytosis of vesicles on the ribbon combined with those at ectopic sites (reviewed in [91]).

The precise cellular and molecular mechanisms underlying ribbon-associated exocytosis are not yet known. As described earlier, the ribbon has been suggested to function like a conveyor belt, moving vesicles toward the active zone in response to depolarization [16]. In potential support of this model, the motor protein KIF3A has been localized to ribbons [30]. However, several pieces of evidence suggest that ribbons act more like a safety belt than a conveyor belt (reviewed in [92]). In particular, the entire releasable pool at the synaptic ribbon can be discharged within 1-2 milliseconds [93,94], which is much faster than the rates that could be achieved with a molecular motor [95]. Furthermore, the addition of ATP-γS to retinal bipolar cell terminals does not affect the initial bout of exocytosis [44], although it does abolish pool refilling. The safety belt model postulates that vesicles are held in close proximity at the ribbon and may undergo compound fusion on this scaffold. Indeed, recent studies have revealed that vesicles are immobilized at bipolar cell ribbons [88], where they may undergo compound fusion in response to a strong stimulus [96]. Compound fusion may be one mechanism through which the ribbon coordinates multivesicular release, which has been reported at hair cell [97] and bipolar cell [98] terminals. Another mechanism for multivesicular release may be the exocytosis of large endosomes, but this occurs with a substantial delay after stimulation [99].

Membrane fusion events are driven by the formation of trans-complexes of SNARE proteins. One membrane contains an R-SNARE/v-SNARE protein such as synaptobrevin/VAMP that provides an alpha helix to the trans-complex. The other membrane contains two Q-SNARE/t-SNARE proteins such as syntaxin and SNAP-25 that contribute a total of three alpha helices to the complex. Specific isoforms of the three core members of the SNARE complex are differentially distributed in ribbon presynaptic terminals (Table 1). For example, syntaxin 1 is present in hair cells [144] and pinealocytes [142,143,162], but absent from retinal ribbon synapses [100,106,111,138,174]. Instead, photoreceptor and bipolar cell terminals express the b isoform [188] of syntaxin 3 [100,102,138,174]. It remains to be determined how syntaxin 3b and other specific SNARE protein isoforms contribute to homotypic and heterotypic vesicle fusion events at ribbon terminals.

At conventional terminals, trans-SNARE complexes appear to be stabilized in a fusion-ready state by complexin 1 or 2 before calcium enters the presynaptic terminal and binds to synaptotagmin 1. This calcium sensor then interacts simultaneously with phospholipid membranes and the assembled SNARE complex to promote fusion (reviewed in [189]). At ribbon synapses, however, the regulation of the calcium-triggering step is poorly understood. Complexins 1 and 2 are replaced by complexins 3 and 4 at ribbon terminals [106,107], where

their functions remain unknown. In addition, the identity of the calcium sensor at these synapses is unclear. Several pieces of evidence suggest that many ribbon terminals utilize a sensor other than a vesicular synaptotagmin (i.e., synaptotagmin 1 or 2). First, 1-2 μM calcium induces tonic exocytosis at photoreceptor [190] and bipolar cell [191] terminals. This calcium concentration is much lower than that needed for binding of synaptotagmin 1 or 2 to syntaxin (half maximal binding at 200 μM). Indeed, synaptotagmin 3 binds syntaxin with much higher affinity (half maximal binding at 1 μM, [192]). Secondly, the sensor for phasic release from MB1 goldfish bipolar cells does not display the calcium-binding affinity of a classical vesicular synaptotagmin (reviewed in [165]). Consistent with this finding, these bipolar cells express synaptotagmin 3 and lack synaptotagmin 1/2 [173]. Third, rat and guinea pig cochlear hair cells lack synaptotagmins 1, 2, 3, and 5. Rather, they express several nonvesicular synaptotagmins—4, 6, 7, 8, and 9—with high calcium affinity [144]. The physiological importance of these synaptotagmins in hair cell synaptic vesicle fusion is not yet known.

Another candidate for the hair cell calcium sensor is otoferlin, encoded by a large gene that is alternatively spliced and translated from several initiation sites [193]. The longest protein contains 6 C2 domains (designated C2A-C2F) homologous to the calcium-binding C2 domains in synaptotagmins and the ferlin family of fusion and membrane repair proteins. Otoferlin's C2 domains bind to SNARE proteins [129] and Cav1.3 [194] in a calcium-dependent manner. Consistent with these interactions and with its robust expression in cochlear hair cells, deletion of exons 14 and 15 (which encode most of the C2C domain) produces transgenic mice with diminished calcium-evoked exocytosis in inner hair cells [129]. These otoferlin-null mice, as well as recently described missense mutants in the C2B [195] and C2F domains [196], lack an auditory brainstem response but maintain normal otoacoustic emissions and vestibular responses. Human patients with mutations in otoferlin share these features of auditory neuropathy, and otoferlin defects are a major cause of nonsyndromic hearing loss in humans [197]. Since otoferlin partially colocalizes with early endosome antigen 1 (EEA1) and GM130, a Golgi protein, in the hair cell cytosol [130], the auditory neuropathy may be due to multiple effects on vesicular trafficking in hair cells.

Mechanisms of endocytosis and vesicle replenishment at ribbon presynaptic terminals

Endocytotic structures appear at the plasma membrane predominantly lateral to active zones (Fig. 4) in photoreceptors [29], bipolar cells [53], pinealocytes [198], hair cells [199], and electroreceptors [14]. Anastomosing tubules [200] and coated vesicles [201] take up extracellular tracers, especially after depolarization [202]. As with exocytosis, capacitance measurements have revealed two distinct kinetic components of endocytosis (reviewed in [203,204]). With a brief stimulus, the fast phase appears with a time constant of 300 milliseconds in mouse cochlear hair cells [205] and 1-2 seconds in goldfish MB1 bipolar cells [206]. With prolonged stimulation of these cells, a slower component appears with a time constant of 15-30 seconds. The fast and slow phases of endocytosis are differentially regulated, suggesting that distinct molecular and cellular mechanisms produce them. For example, high calcium selectively triggers the fast phase in mouse inner hair cells [207], and hydrostatic pressure differentially inhibits the slow phase in bipolar cells [208].

Despite intense interest over the past 15 years, the cellular and molecular mechanisms that contribute to the fast and slow components of endocytosis are poorly understood. One possible mechanism for the fast component is kiss-and-run, where vesicles interact transiently with the plasma membrane to make a fusion pore. However, kiss-and-run was not observed in bipolar cells utilizing total internal reflection fluorescence microscopy [209] or interference reflection microscopy [210]. Another possible mechanism for the fast mode is bulk endocytosis, whereby large, uncoated invaginations pinch off from the plasma membrane in response to a strong

stimulus. At conventional terminals, these bulk endosomes form 1-2 seconds after stimulation [211]. Anastomosing tubules and large endosomes have long been appreciated as important endocytotic structures at hair cell [199,212] and goldfish bipolar cell [213,214] terminals, but it remains to be determined whether they appear rapidly after stimulation. Bulk endocytosis may have an early or intermediate role in vesicle retrieval at ribbon terminals since synaptic vesicles bud from internalized endosomes to re-enter the releasable pool [212,214].

A third possible mechanism for vesicle retrieval at ribbon terminals is clathrin-mediated endocytosis (CME), which begins with the recruitment of adaptor proteins such as AP2, AP180, and amphiphysins to the plasma membrane (reviewed in [215]). Clathrin triskelia form a coated pit around a progressively invaginating vesicle that is ultimately severed from the plasma membrane via the GTPase activity of dynamin. Uncoating of the synaptic vesicle occurs through the enzymatic activities of synaptojanin, among other proteins. Consistent with a role for CME in vesicle retrieval, many components of the pathway have been found at retinal ribbon terminals (Table 1). For example, Sherry and Heidelberger [103] localized clathrin, amphiphysin, and dynamin to photoreceptors and bipolar cells, although dynamin was only highly expressed in mouse rod bipolar cell terminals. Retina-specific isoforms of unknown function have been identified for amphiphysin I [102,216] and dynamin 1 [217]. Among other major players in endocytosis, AP180 [104] and synaptojanin [8] are enriched at retinal ribbon terminals. In striking confirmation of its importance in the vesicle cycle, zebrafish with a truncation mutation in synaptojanin1 lack an optokinetic response and have abnormal ERGs [8,218,219]. Cone, but not bipolar cell, terminals from these mutants harbor several defects, including 50% fewer synaptic vesicles, 57% fewer anchored ribbons, and a 10-fold increase in endosomal area. These results suggest that synaptojanin and possibly other clathrin pathway components regulate endocytosis at some retinal ribbon terminals.

Is the clathrin pathway responsible for the slow or fast mode of endocytosis? Perturbation of CME with polypeptides directed against either clathrin, AP2, amphiphysin I or II, or dynamin reduced the slow, but not the fast, component of endocytosis at goldfish bipolar cell terminals [220]. Inhibition of GTP hydrolysis also perturbed the slow component. Other studies, however, present evidence suggesting that clathrin [214] and GTP hydrolysis [44,221] do not contribute to endocytosis at these terminals. Rather, the latter studies revealed a requirement for ATP hydrolysis in compensatory endocytosis. These discrepancies highlight the need for additional studies to determine the morphological and molecular bases of the slow and fast components of endocytosis at ribbon terminals.

Ribbon-associated vesicles can contain extracellular tracers such as horseradish peroxidase [201], suggesting that these vesicles are in the endocytotic pathway. Replenishment of ribbons can be extraordinarily fast in cone photoreceptors [222], thereby supporting tonic exocytosis for prolonged periods of time. How do synaptic vesicles traffic to the ribbon? At present, the molecular and cellular mechanisms are poorly understood. The large cytoplasmic pool of vesicles found in most ribbon terminals is more mobile than at conventional synapses [88, 222], possibly due to the absence of synapsins [148,223]. Vesicles may move rapidly with the assistance of one or more unconventional myosins, although direct evidence for this mechanism is still lacking. However, photoreceptors in mice with a mutation in myosin Va have partially denuded ribbons with ectopic clusters of synaptic vesicles in the terminals [224]. Abnormal ERG b-waves are present in these mice, as well as in mice with mutations in myosin VI [225] and myosin VIIa [226]. Patients with mutations in myosin VIIa suffer from Usher syndrome type IB, characterized by congenital deafness and vestibular defects in addition to retinal degeneration (reviewed in [227]). Since myosin VIIa is present in several domains in receptor cells besides the presynaptic terminal, it is currently unclear to what extent synaptic vesicle trafficking contributes to the disorder (see, for example, [228]).

VGLUT1 and VGLUT3 fill synaptic vesicles with glutamate at ribbon terminals

Glutamatergic vesicles become available for reuse following refilling by VGLUTs, a family composed of three structurally related vesicular neurotransmitter transporters with a largely complementary distribution pattern [229]. In the retina, VGLUT1 localizes exclusively to the ribbon terminals of photoreceptors and bipolar cells, while VGLUT2 is found in ganglion cells and 10% of cone pedicles (Table 1). The importance of VGLUT1 in visual transduction is underscored by the absence of visual evoked potentials in the visual cortex of VGLUT1 knockout mice [230]. Synaptic transmission throughout the outer plexiform layer is impaired given the absence of an ERG b-wave under either scotopic or photopic conditions. Interestingly, VGLUT1 is expressed in most (if not all) pinealocyte synaptic-like microvesicles and co-localizes with VGLUT2 in a subset of them [166,231]. An alternatively spliced VGLUT1 isoform, with a 25 amino acid insert of unknown function in the first intravesicular loop, constitutes 70% and 25% of VGLUT1 mRNA in the adult retina and pineal, respectively [232]. Retinal and pineal ribbon terminals may therefore share common mechanisms for loading glutamate into their vesicles.

While VGLUT1 and VGLUT2 are expressed at terminals that release glutamate, VGLUT3 is primarily expressed by interneurons that release other neurotransmitters [233]. Consistent with this hypothesis, retinal expression of VGLUT3 is confined to a subpopulation of glycinergic amacrine cells [151,181,182]. Until recently, it was not known if strictly glutamatergic neurons could express VGLUT3. Several reports published last year revealed that hair cells, in fact, utilize VGLUT3 as their primary vesicular transporter. Zebrafish [234], humans [172], and mice [132,172] with VGLUT3 mutations exhibit profound deafness. Intact cochlear sound amplification suggests normal outer hair cell function [132]. The primary defect occurs at the afferent presynaptic terminals of inner hair cells, as whole cell recordings from auditory nerve fiber terminals reveal postsynaptic responses with kainate but not after depolarization with high levels of potassium. Capacitance measurements do not reveal a defect in the kinetics or amount of vesicle fusion [172], although the vesicle pool near the ribbon is smaller in the zebrafish mutants [234] but not the mouse mutants [132]. Interestingly, the zebrafish, but not mouse, mutants also exhibit balance defects. Taken together, these results suggest that VGLUT3 is essential for loading glutamate into synaptic vesicles in some populations of hair cells.

Calcium buffering, sequestration, and release from internal stores

The precise regulation of the synaptic vesicle cycle relies heavily on the intra-terminal calcium landscape, with peaks and valleys shaped by multiple buffers and stores (Fig. 5). Pioneering work by Roberts [235,236] revealed that mobile buffers bind calcium within a few microseconds of entry into frog saccular hair cells, thereby limiting spatiotemporal spread of the exocytotic signal. Several calcium-binding proteins have been proposed to serve as mobile buffers in hair cells, including calbindin [236], calretinin [237], and parvalbumin 3 [238]. At retinal ribbon synapses, the function of these calcium-binding proteins is unclear, especially given the morphologically normal retina of calbindin knockout mice [239].

Endoplasmic reticulum (ER) in the terminals of photoreceptors [240], bipolar cells [241], pinealocytes [242], and hair cells [243] sequesters calcium presumably via sarcoplasmic-endoplasmic reticulum calcium ATPases (SERCAs; Fig. 5). These pumps comprise a family of three genes that are alternatively spliced to produce several proteins that transport calcium from the cytosol into the ER lumen. SERCA2 is the predominant isoform in photoreceptor and bipolar cell terminals [244,245] and localizes very close to ribbons [246]. While several studies have implicated one or more SERCAs in hair cells through the use of inhibitors such as thapsigargin and cyclopiazonic acid (see, for example, [247,248]), its identity is unknown.

The outer hair cell subsynaptic cistern, which is located within 20-30 nm of the plasma membrane directly across from efferent terminals, has long been suspected to be a calcium store given its resemblance to muscle sarcoplasmic reticulum (reviewed in [249]). The efferent presynaptic terminal releases acetylcholine onto the outer hair cell, inducing calcium influx and subsequent activation of SK channels. The outer hair cell then hyperpolarizes, thereby inhibiting the cochlear amplifier (reviewed in [250]). Calcium-induced calcium release (CICR) from intracellular stores regulates this efferent feedback since exogenous ryanodine or caffeine can modulate otoacoustic emissions [251]. These two drugs primarily target the ryanodine receptor (RyR) calcium release channels, which are homotetramers of three homologous proteins, on the endomembrane. Lioudyno et al. [252] have proposed that RyR1 on the subsynaptic cistern couples efferent input with CICR in order to regulate the cochlear amplifier.

Release of calcium from intracellular stores also modulates exocytosis at several ribbon terminals. Inner hair cells, which express RyR1 and RyR2 [253,254], modify afferent nerve fiber activity in response to exogenous ryanodine [253]. Calcium release from RyR-gated [255,256] and inositol 1,4,5-trisphosphate receptor (IP_3R)-gated [255] stores potentiates exocytosis from vestibular hair cells during prolonged stimulation. Similarly, prolonged stimulation of rods releases calcium from ryanodine-sensitive stores to boost and maintain exocytosis [257-260]. Since rods tonically release neurotransmitter in the dark at a resting membrane potential where most of their voltage-gated calcium channels are closed, CICR ensures that exocytosis occurs under physiologic conditions [260]. The RyR that mediates CICR in rods is unknown; however, RyR2 has been found in photoreceptor terminals [244, 258].

Ribbon presynaptic terminal calcium stores have recently been implicated in another pathway that maintains intracellular calcium levels and exocytosis. Szikra et al. [261] identified store-operated calcium entry (SOCE), possibly through TRPC1, as a requirement for light-adapted rod terminals to maintain exocytosis. Since rods continue to release neurotransmitter even under saturating white light conditions [262], calcium levels need to be maintained via a voltage-independent mechanism such as SOCE. TRPC-mediated entry also occurs in hair cells to maintain intracellular calcium levels [263]. Thus, CICR from the ER and store refilling through calcium influx pathways are important in calcium homeostasis and in supporting tonic exocytosis at several ribbon terminals.

Besides ER, mitochondria are known to sequester calcium (reviewed in [264]). Indeed, mitochondria frequently appose the ER and may interact to regulate calcium homeostasis (reviewed in [265]). In bipolar cells, however, calcium uptake into mitochondria was only observed with high intracellular calcium levels [266]. This study demonstrated that the principal role for mitochondria in these terminals is to generate large quantities of ATP. In potential support of a minor role for mitochondria in calcium sequestration at some ribbon terminals, studies have revealed that mitochondria cluster far from ribbons in cone [265], bipolar cell [90], and inner hair cell (reviewed in [249]) terminals.

Plasma membrane calcium-ATPases extrude most of the calcium from ribbon terminals

To prevent an overload of calcium in the terminal, extrusion must eventually occur into the extracellular space. This is especially important for ribbon terminals where slowly inactivating calcium channels allow for the accumulation of large intracellular calcium loads. To maintain neurotransmitter release, however, calcium levels must remain high near active zones, suggesting spatial regulation for extrusion. Two major calcium extrusion mechanisms exist: plasma membrane calcium-ATPases (PMCA) and sodium-calcium exchangers (NCX). PMCA appears to be the dominant mechanism for clearing calcium from most ribbon terminals

[266-268]. Inhibition of PMCA with sodium orthovanadate in photoreceptors [268] or bipolar cells [266] maintains high intracellular calcium levels. PMCA segregates away from ribbon-associated active zones by localizing to the lateral walls of terminals [268] via interactions with a protein complex that includes PSD95, Veli3, and MPP4 [246]. Indeed, genetic ablation of MPP4 results in the loss of PMCA from the presynaptic plasma membrane and altered calcium homeostasis [246].

In mammals, the PMCA family contains four genes that are alternatively spliced at two main sites (termed A and C) to generate isoforms that may have specific local functions. In hair cells, a large insert in the A site targets the PMCA isoform to the apical stereociliary bundles, while a Leu-Ile motif in the C site provides a targeting signal to the basolateral domain [269,270]. Most PMCA1 isoforms appear to contain the Leu-Ile motif [271], so they may regulate basal calcium levels near ribbons. PMCA2, on the other hand, is targeted primarily to the apical stereociliary bundles [269,270]. Deafwaddler mice have mutations in PMCA2 that diminish pump activity, leading to hearing and balance defects [272]. These mice also exhibit ERG b-waves with decreased amplitudes and slow kinetics [273], suggesting a defect in photoreceptor synaptic transmission. Evidence for PMCA2 involvement in the rod pathway was obtained from recordings of light responses from Deafwaddler rod bipolar cells, which revealed a 50% decrease in sensitivity. Since PMCA1 is expressed in rod and cone terminals [274], it will be important to determine whether PMCA1 has non-redundant functions in the OPL. Taken together, these results suggest that specific PMCA isoforms localize to specific niches in sensory neurons and play a major role in clearing calcium from their presynaptic terminals.

While PMCA may be the dominant calcium extrusion mechanism at ribbon terminals, NCX has been proposed to promote calcium extrusion from cones [265], rod bipolars [274], and mixed rod-cone bipolars [275]. Johnson et al. [265] suggest that the low affinity/high turnover NCX could potentially decrease calcium levels rapidly when cones are stimulated with light. The high affinity/low turnover PMCA could maintain a low level of calcium in rods during darkness. The utilization of these two extrusion mechanisms, along with sequestration, buffering, and intake through L-type calcium channels in a spatiotemporally regulated manner, maintains calcium homeostasis. This precise regulation of calcium signaling supports efficient synaptic vesicle cycling and the extraordinary performance of ribbon presynaptic terminals.

Conclusions

In this review, we have described the organization and function of some of the molecular constituents of ribbon presynaptic terminals. The molecular architecture of ribbon synapses resembles that of conventional synapses despite their ultrastructural differences. So far, only RIBEYE appears to be unique to ribbon terminals, probably because it is the major structural component of the ribbon itself. However, particular ribbon synapses appear to utilize specific isoforms of synaptic proteins to fit their physiological needs. In several instances, mouse and zebrafish mutants have provided strong evidence for the involvement of specialized isoforms in the rapid and tonic synaptic transmission found in the visual, vestibular, and auditory systems.

Several major questions remain unanswered. First of all, the functions of RIBEYE and other ribbon components continue to be enigmatic. Bassoon mutant mice lack the fast, but not the slow, component of exocytosis in inner hair cells without properly anchored ribbons. The presence of slow exocytosis in these mutants does not preclude the possibility that the ribbon sustains neurotransmitter release under normal physiological conditions. Therefore, additional studies are needed to clarify the roles of the ribbon in exocytosis. In addition, the molecular components of the ribbon, vesicle-associated tethers, and arciform density should be delineated further. What signals direct the assembly of the ribbon and its surrounding domains?

Cytomatrix proteins may be involved, but most of their roles have not yet been defined. Multiple modes of exocytosis and endocytosis exist at ribbon terminals, yet their molecular and cellular mechanisms are mostly unclear. While progress has been made in defining the composition of synaptic vesicles at ribbon terminals, the molecular signatures of the different vesicle pools are unknown. Finally, continued investigation into the spatiotemporal regulation of calcium buffering, sequestration, and release may shed light on these important aspects of calcium homeostasis. The elucidation of these and other remaining questions about the molecular architecture of ribbon synapses should provide new insights into the pathophysiology of synaptopathies.

Acknowledgments

This work was supported by National Institutes of Health Grants F30 NS061494-01 (G.Z.) and R01 EY003821 (G.M.).

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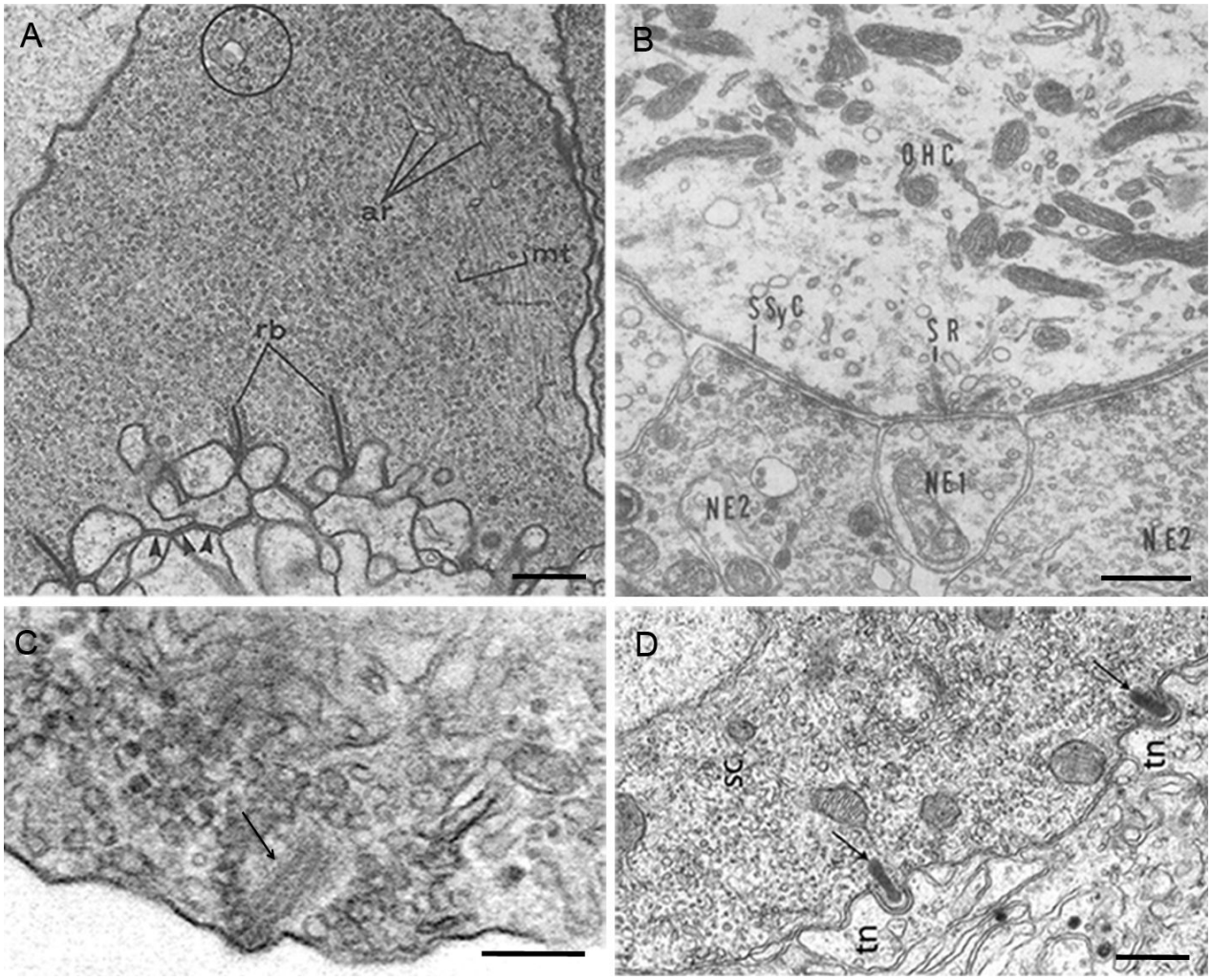

Fig. 1.

Examples of ribbon presynaptic terminals. In panel A, an electron micrograph of a dark-adapted, turtle cone presynaptic terminal is shown. Postsynaptic processes are apposed to ribbons (rb) and basal junctions (arrowheads) in the terminal. The large population of synaptic vesicles is a hallmark of most ribbon presynaptic terminals. Other organelles in this terminal include microtubules (mt), agranular reticulum (ar) and vacuoles (circle). Figure modified from (4), with permission of The Rockefeller University Press (copyright 1978). Panel B shows an electron micrograph of the basal portion of a guinea pig outer hair cell (OHC), which makes contact with afferent (NE1) and efferent (NE2) nerve fibers. Two synaptic ribbons (SR) are directly apposed to the afferent nerve fiber, while subsynaptic cisterns (SSyC) are opposite the efferent nerve fibers. A relative paucity of synaptic vesicles and abundant mitochondria also characterize the OHC ribbon terminal. Figure modified from (5), with permission of Elsevier (copyright 1980). In panel C, an electron micrograph shows a goldfish bipolar cell synaptic ribbon (arrow) at high magnification. Three distinct laminae can be observed in this ribbon. Panel D reveals an electron micrograph of an elephantfish promormyromast. These sensory organs of the lateral line contain electroreceptors (sc) that synapse onto terminal neural boutons (tn). Ribbons (arrows) nestle within invaginating synapses. Also present in the electroreceptor terminal are numerous synaptic vesicles and mitochondria. Figure modified from (6), with permission of John Wiley and Sons (copyright 2007). Scale bars, 0.5 μm (A, B and D), 0.15 μm (C).

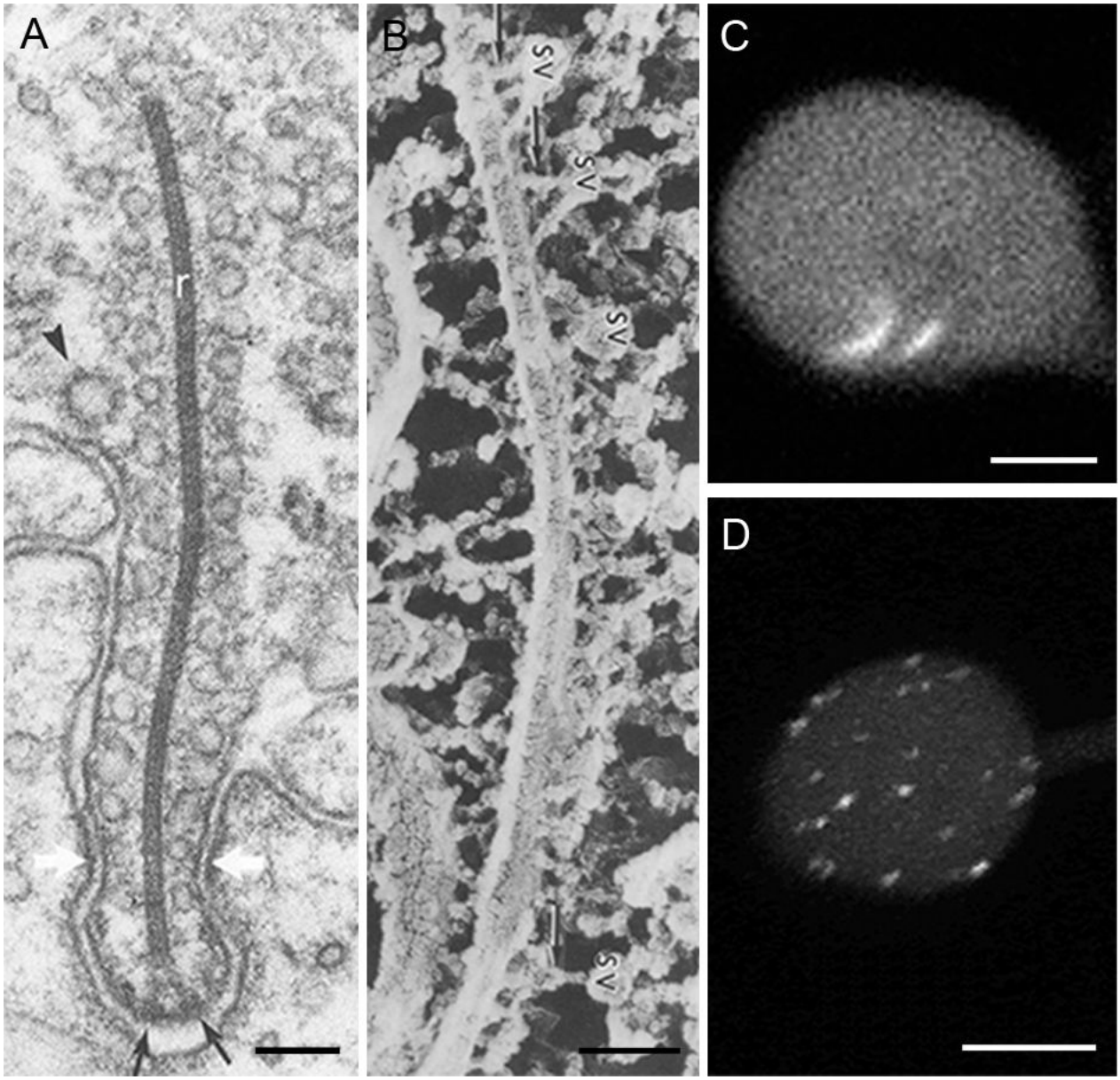

Fig. 2.

Structure of the synaptic ribbon. Panel A contains an electron micrograph of a skate electroreceptor ribbon synapse. The ribbon (r), which appears electron-dense and laminar, is located in an evagination of the presynaptic plasma membrane flanked by postsynaptic processes. Vesicles are attached to the surface of the ribbon except at its base, which is connected to osmiophilic aggregates on the plasma membrane (black arrows). Postsynaptic densities are most prominent adjacent to the constrictions in the presynaptic plasma membrane (white arrows). Coated vesicles (an example is marked by the arrowhead) are found lateral to the ribbon and its active zones. Figure modified from (14), with permission of Chapman and Hall (copyright 1982). Panel B shows a freeze-etched replica of a cross-fractured frog photoreceptor synaptic ribbon and its surrounding environment. Synaptic vesicles (SV) are tethered to the ribbon by filaments (arrows). Figure modified from (15), with permission of Springer (copyright 1987). In panel C, a confocal micrograph of a goldfish cone presynaptic terminal filled with a fluorescent RIBEYE-binding peptide is shown. Ultrastructural analysis

confirmed that the two long, curvilinear structures were synaptic ribbons (data not shown). Panel D shows several ribbons in a 3D reconstruction from optical sections through the synaptic terminal of a goldfish bipolar cell dialyzed via a whole-cell patch pipette with the fluorescent RIBEYE-binding peptide. The smaller size and greater number of puncta in the bipolar cell are consistent with the characteristics of synaptic ribbons in this cell. Scale bars, 0.1 μm (A and B), 2.5 μm (C), 5 μm (D).

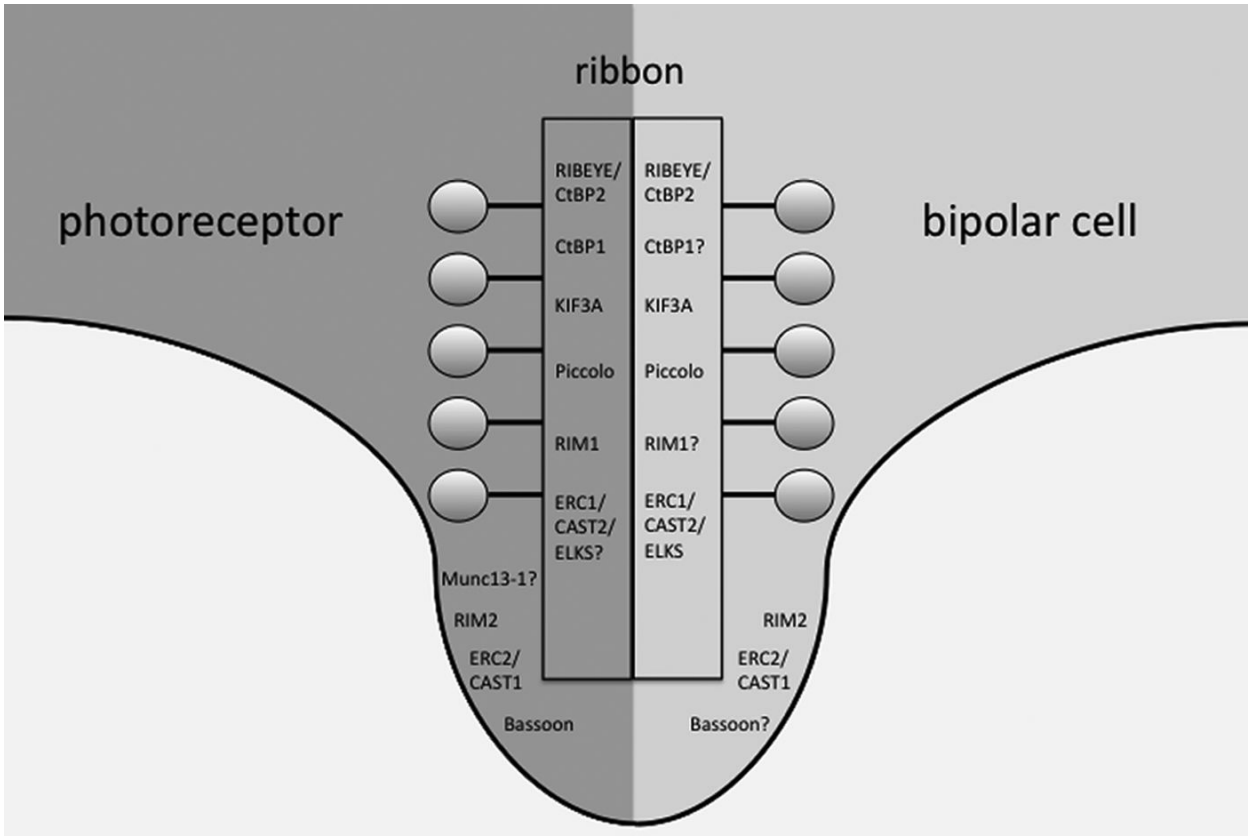

Fig. 3.

Cytomatrix proteins identified at the synaptic ribbon or the active zone in retinal ribbon terminals. The left side of the schematic contains those proteins localized to photoreceptor terminals, while the right side reveals their identity in bipolar cell terminals. A question mark denotes either a discrepancy in the literature or that the protein localization has not been determined.

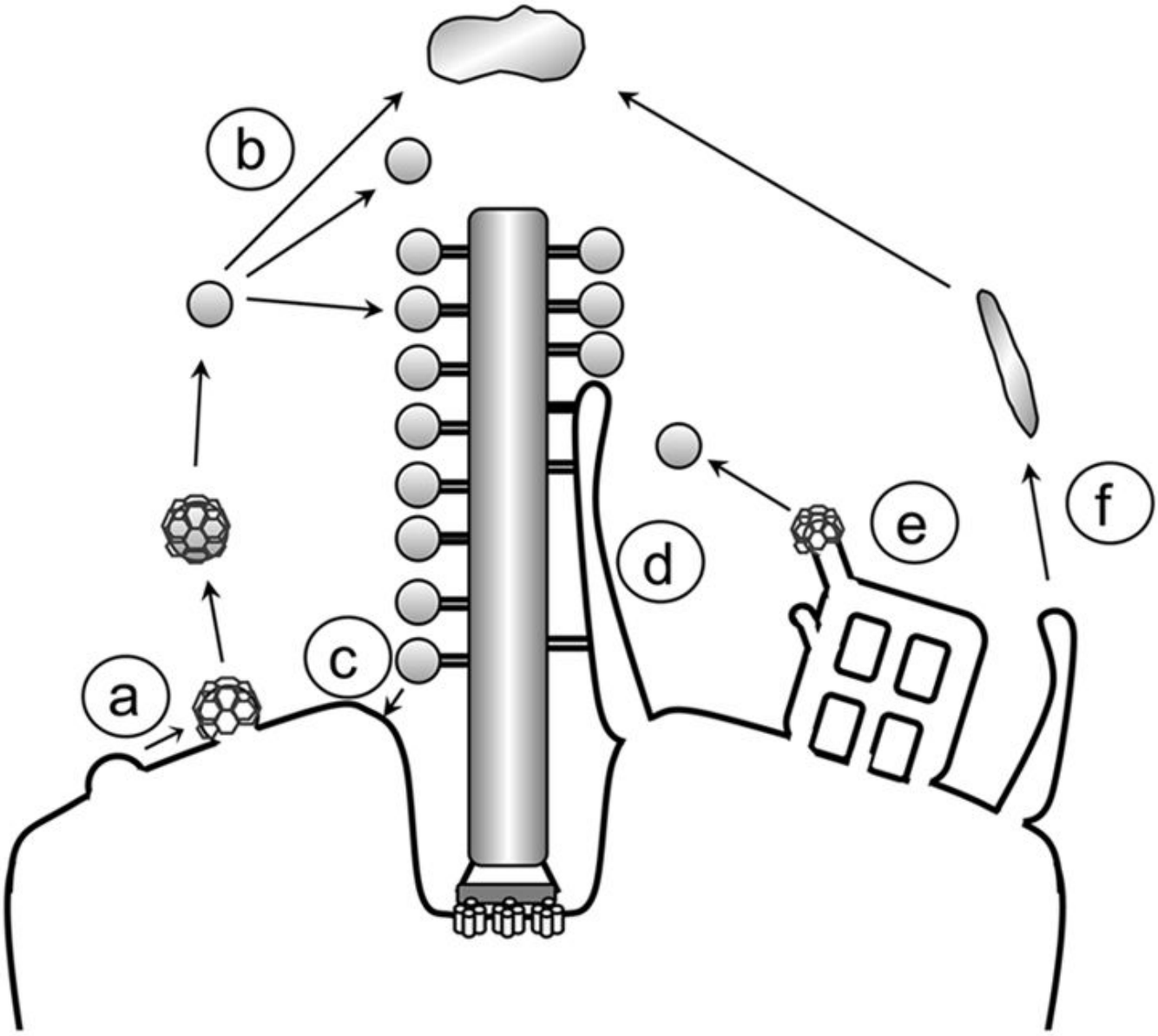

Fig. 4.

Events in the synaptic vesicle cycle near the ribbon. Clathrin-mediated endocytosis (A) retrieves vesicles that can either coalesce with a presynaptic cistern (B, top arrow) or enter either the reserve (middle arrow) or releasable (bottom arrow) pools. Single (C) or multiple (D) ribbon-associated vesicles fuse with the plasma membrane lateral to L-type voltage-gated calcium channels. Vesicle retrieval may also occur from large anastomosing tubules (E) or directly via large endosomes (F).

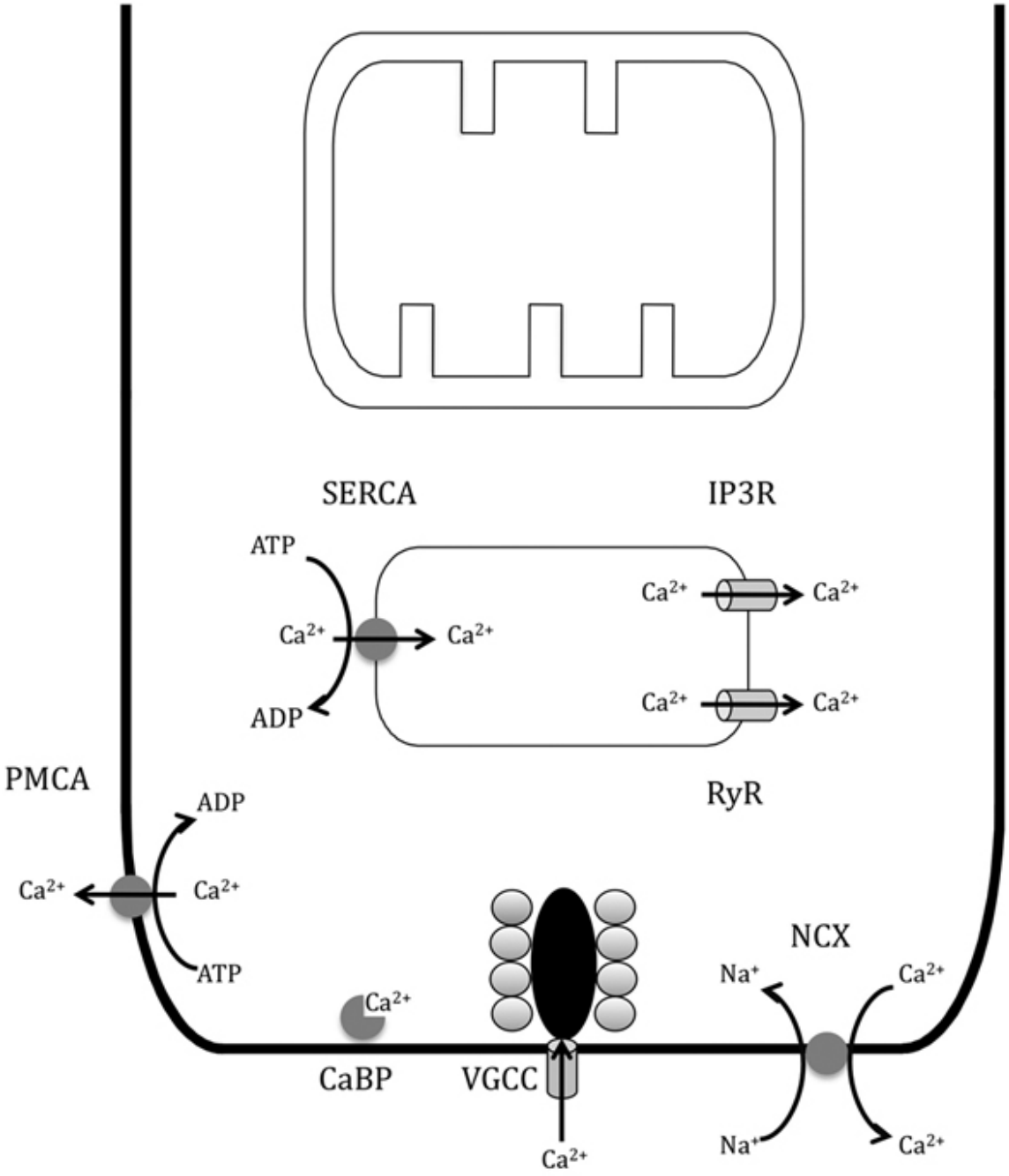

Fig. 5.

Calcium handling mechanisms at ribbon presynaptic terminals. Calcium influx occurs through L-type voltage-gated calcium channels (VGCC) found below the ribbon. Mobile calcium-binding proteins (CaBP) quickly limit the spread of calcium, which is also sequestered into endoplasmic reticulum (ER) by sarcoplasmic ATPases (SERCAs). Activation of ryanodine receptors (RyR) or inositol 1,4,5-trisphosphate receptors (IP3R) induces release of calcium from ER stores through these channels. Calcium is primarily extruded from ribbon presynaptic terminals via plasma membrane calcium ATPases (PMCA) located on the lateral walls of the terminal, but may also be extruded by the sodium-calcium exchanger (NCX).

Table 1

Expression of synaptic vesicle cycle proteins in adult ribbon presynaptic terminals

Protein	Photoreceptor	Bipolar cell	Pinealocyte	Hair cell
Amphiphysin	Yes [100-103]	Yes [101-103]	?	?
AP180	Yes [104]	?	?	?
Clathrin	Yes [100,103,105]	Yes [103]	?	?
Complexin 1	No [106,107]	No [106,107]	?	?
Complexin 2	No [106,107]	No [106,107]	?	?
Complexin 3	Yes [107]	Yes [107]	?	?
Complexin 4	Yes [107]	Yes [107]	?	?
CSP	Yes [108]	?	Yes [109]	Yes [108,110]
Dynamin	Yes [103,111]	Yes [103]	Yes [112]	?
GLT1/EAAT2	Yes [113-120], No [121,122]	Yes [113-118,120-122]	Yes [123,124]	?
Munc13	Yes [23], No [125]	No [125]	Yes [27]	?
Munc18	Yes [111]	?	Yes [109,112]	?
Munc119/RG4	Yes [126,127]	No [126,127]	?	?
NSF	?	?	Yes [128]	?
Otoferlin	?	?	?	Yes [129-133]
Rab3a	Yes [100,111], No [134]	No [134]	Yes [135]	Yes [136]
Rabphilin	Yes [100], No [100]	?	?	?
SNAP-23	Yes [100]	?	?	?
SNAP-25	Yes [100,111, 137-140], No [134, 141]	Yes [137,138], No [134, 141]	Yes [112,142, 143]	Yes [110,144]
SV2	Yes [100,140, 145-149], No [146]	Yes [140,146,148-151], No [146]	Yes [152], No [152]	No [153]
Synapsin 1	Yes [100], No [100, 106,137,140,141,148, 150,154,155]	No [106,137,140,150,154]	No [156,157]	Yes [158,159], No [153]
Synapsin 2	No [148]	No [148]	?	?
Synaptobrevin/VAMP	Yes [100,139,140, 150,160,161]	Yes [140,150,160]	Yes [142,143, 157,162], No [162]	Yes [144,153]
Synaptophysin 1/2	Yes [100,111,137, 140,141,147,148,155, 163,164]	Yes [137,141,148,165]	Yes [112,128, 142,143,156,162, 166,167]	Yes [136,159, 168-170], No [110,144,171, 172]
Synaptotagmin 1/2	Yes [100,111,163, 165,173], No [163, 165,173]	Yes [163,173], No [163,165, 173]	Yes [142,143, 157,162]	No [144]
Synaptotagmin 3	?	Yes [173], No [173]	?	?
Syntaxin 1	No [100,102,106,111, 138,174]	No [106,138,174]	Yes [142,143, 162]	Yes [144]
Syntaxin 2	No [174]	No [174]	?	?
Syntaxin 3	Yes [100,102,138, 174]	Yes [102,138,174]	?	?
Syntaxin 4	No [174]	No [174]	?	?

Protein	Photoreceptor	Bipolar cell	Pinealocyte	Hair cell
V-ATPase	?	?	Yes [128,157]	?
VGAT/VIAAT	No [175,176]	Yes [177], No [175,176]	Yes [178]	?
VGLUT1	Yes [107,149,150, 164,179-185]	Yes [107,149-151,164,177, 179-182,184,185]	Yes [166]	Yes [186], No [132,187]
VGLUT2	Yes [182,183], No [149,164,179,185]	No [149,164,179,182,183, 185]	Yes [166,167]	No [132,186, 187]
VGLUT3	No [151,164,181,182, 185]	No [151,164,181,182,185]	?	Yes [132,187]

AP assembly protein, *CSP* cysteine string protein, *GLT* glutamate transporter, *EAAT* excitatory amino acid transporter, *Munc* mammalian UNC, *RG* retinal gene, *NSF* N-ethylmaleimide-sensitive factor, *SNAP* synaptosome-associated protein, *SV* synaptic vesicle, *VAMP* vesicle-associated membrane protein, *VGAT* vesicular GABA transporter, *VIAAT* vesicular inhibitory amino acid transporter, *VGLUT* vesicular glutamate transporter, *?* expression not yet determined