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Review

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Presynaptic active zones in invertebrates and vertebrates

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Abstract

The regulated release of neurotransmitter occurs via the fusion of synaptic vesicles (SVs) at specialized regions of the presynaptic membrane called active zones (AZs). These regions are defined by a cytoskeletal matrix assembled at AZs (CAZ), which functions to direct SVs toward docking and fusion sites and supports their maturation into the readily releasable pool. In addition, CAZ proteins localize voltage-gated Ca^{2+} channels at SV release sites, bringing the fusion machinery in close proximity to the calcium source. Proteins of the CAZ therefore ensure that vesicle fusion is temporally and spatially organized, allowing for the precise and reliable release of neurotransmitter. Importantly, AZs are highly dynamic structures, supporting presynaptic remodeling, changes in neurotransmitter release efficacy, and thus presynaptic forms of plasticity. In this review, we discuss recent advances in the study of active zones, highlighting how the CAZ molecularly defines sites of neurotransmitter release, endocytic zones, and the integrity of synapses.

Keywords active zone; cytoskeletal matrix; fusion; release; synaptic vesicle
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See the Glossary for abbreviations used in this article.

Introduction

Chemical synapses are asymmetric structures comprising a presynaptic bouton, designed for the rapid and regulated release of neurotransmitter, and a postsynaptic neurotransmitter reception apparatus (the postsynaptic density—PSD) separated by a small space known as the synaptic cleft. In general, synapse size and morphology are closely coupled to function and reliability [1,2]. For example, reliable synapses, such as the neuromuscular junction (NMJ), tend to be very large, containing thousands of SVs and elaborate AZs and PSDs, thereby ensuring that muscle contraction occurs with high fidelity [3]. Conversely, synapses of the central nervous system, such as hippocampal glutamatergic synapses, are generally small and less reliable, yet possess an enormous capacity to change their reliability/strength in a process called synaptic plasticity. Importantly, synaptic plasticity can involve changes on either side of the synapse, including alterations in neurotransmitter release probability (presynaptic) or in the number and activity of postsynaptic neurotransmitter receptors (postsynaptic). In the current review, we will highlight roles of CAZ proteins in regulating the fidelity, reliability, plasticity, and integrity of synapses, drawing upon advances at invertebrate and vertebrate synapses.

Invertebrate active zones

Active zones in Caenorhabditis elegans

Active zones of NMJ synapses in the worm *Caenorhabditis elegans* (*C. elegans*) comprise a broad surface of plasma membrane situated between electron-dense projections (DPs) and flanking cellular tight junctions [4]. At these synapses, most SV fusion and endocytic events occur within 30–200 nm of the DPs, suggesting a role for these structures in the regulated release of neurotransmitter (Fig 1) [1]. One of the first components identified at these AZs was SYD-2, a multidomain scaffold protein that is structurally related to the vertebrate presynaptic protein Liprin-α [5–7]. Loss-of-function mutations in Syd-2 cause AZs to become less compact and more elongated [5], and impair SV docking and synaptic transmission, indicating that SYD-2 organize the neurotransmitter release machinery [8,9]. Consistent with these findings, SYD-2 and Liprin-α have been found to interact and colocalize with other CAZ proteins including ELKS-1, UNC-10/RIM, and UNC-13 at the *C. elegans* NMJ. These molecules are also required for the regulated docking and fusion of SVs at *C. elegans* NMJ synapses. For example, loss of UNC-10/RIM not only impairs synaptic transmission, but also causes a redistribution of docked vesicles away from the DPs [10]. This finding suggests that UNC-10/RIM may position SVs close to sites of calcium entry via voltage-gated calcium channels (VGCCs) [8] (Table 1).

Greater insight into the 3D organization of SV pools associated with *C. elegans* AZs has come with the development of high-pressure freeze fixation techniques (hpf-EM) combined with electron tomography [8,9]. These unprecedented images reveal that SVs, clustered within NMJ boutons, are not freely floating but rather interconnected by a fine filamentous network [8] similar to that seen at vertebrate synapses (Fig 1) [11,12]. These filaments are also

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Glossary

Abp	actin-binding protein 1
AP2	adapter protein 2
APC	anaphase-promoting complex
AZ	active zone
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BRP	Bruchpilot
CAST	CAZ-associated structural protein
Cav2.1	P/Q voltage-dependent calcium channel
CAZ	cytomatrix at the active zone
Cdc42	cell division control protein 42
CME	clathrin-mediated endocytosis
CtBP	C-terminal-binding protein 1
Daam	Disheveled-associated activator of morphogenesis 1
DH	Dbl Homology
DP	dense projections
Dunc13	Drosophila homolog of unc13
EH	Eps15 homology
ELKS	protein rich in the amino acids E, L, K, and S
EM	electron microscopy
ERCs	ELKS-Rab6-interacting protein CAST
FboX45	F-box protein 45
GEF	guanine exchange factor
GIT1	ARF GTPase-activating protein 1
Hpf	high-pressure freezing
ITSN-L	intersectin-long
ITSN-S	intersectin-short
kDa	kilodalton
Kif3A	kinesin-like protein 3A
Nlg	neuroligin
NMJ	neuromuscular junction
Nrx	neurexin
PDZ	postsynaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PH	pleckstrin homology
PP2A	protein phosphatase 2A
PSD	postsynaptic density
Pvr	vesicular release probability
Rab3A	synaptic 21-kDa GTP-binding protein
RBP	RIM-binding protein
RIM	Rab3-interacting molecule
SH3	Src homology domain 3
Siah1	seven in absentia homolog 1
SNAP	synaptosomal-associated protein
SNARE	soluble *N*-ethylmaleimide-sensitive-factor attachment receptor
SV	synaptic vesicle
Syd1	synaptic scaffold protein 1
Unc13	uncoordination mutant 13
UPS	ubiquitin–proteasome system
VAMP-2	synaptobrevin-2
VGCC	Voltage-gated calcium channel
Wrd	Well rounded
Zn	Zinc
ZnF	Zinc finger

observed between SVs and the AZ plasma membrane [8]. At present, little is known about the structural organization of vesicle clusters in *C. elegans,* or of the proteins building these filamentous networks, though SYD-2/Liprin-α and UNC-10/RIM are possible candidates. This latter concept is supported by experiments showing that in *syd-2* and *unc-10* loss-of-function mutants, the number of docked vesicles contacting DP filaments is reduced, as is evoked neurotransmission [8,9]. Hpf-EM has also revealed that AZs in *C. elegans* are assembled through the polymerization of these DPs into 2D arrays that scale with SYD-2 expression levels and synaptic transmission, suggesting that SYD-2-dependent changes in AZ size may be part of a plasticity mechanism used to adjust the reliability of synaptic transmission in *C. elegans* [9]. These features imply that neurotransmitter release sites in general are organized in modules that can be assembled into a diverse set of arrays with distinct functionalities, as will be discussed below.

Drosophila active zones

Active zones present at the *Drosophila melanogaster* NMJ are more elaborate than those observed in *C. elegans* [1,13]. They have been given the name "T-bars", reflecting their morphology as a meshwork of filaments overlying a pedestal (Fig 1) [14]. Initial studies identified the protein Bruchpilot (BRP) as a key component of the T-bar [15], as this structure is lacking in *brp* null mutants [16]. Super-resolution microscopy further revealed that single BRP molecules adopt an elongated conformation, reaching from the AZ membrane into the cytoplasm of boutons, and thereby forming the T-bar structure (Fig 1). In addition, BRP is essential for the clustering of Ca^{2+} channels beneath the T-bar at the center of the AZ [17], bringing the Ca^{2+} source close to the fusion machinery. This close association is created by two BRP isoforms that assemble in an alternating pattern, forming a circular array around the T-bars. This arrangement appears to create discrete adjacent slots for the Ca^{2+} channels and SV docking sites that are critical for efficient neurotransmission [18].

In addition to Bruchpilot, T-bars also contain RIM, Dunc13, RBP, and Fife. Rab3-interacting molecule (RIM) has long been appreciated for its role in synaptic transmission, working in concert with the *Drosophila* homolog of Unc13, Dunc13, to promote SV priming and docking [19]. In *Drosophila*, RIM loss-of-function decreases synaptic transmission by reducing neurotransmitter release probability without affecting the morphology/integrity of AZs [19], an observation consistent with normal levels of BRP at synapses lacking RIM. Intriguingly, RIM loss-of-function also reduces the synaptic localization of cacophony, a VGCC, explaining in part the reduced release probability at synapses lacking RIM [19].

Coupling of VGCCs to AZs is also mediated by RIM-binding proteins (RBPs) of which there are three variants in mammals and one in *Drosophila* [20,21]. As with RIM, RBP loss-of-function impairs synaptic transmission at the *Drosophila* NMJ and causes the mislocalization of cacophony [21]. Here, RBP appears to perform a corralling function that surrounds and maintains VGCC beneath T-bars, a concept consistent with a reduction in electron density at the base of T-bars in synapses lacking RBP [21]. Importantly, the tufts created by BRP still form without RBP or RIM, implying that as in *C. elegans*, the functional assembly of AZs is hierarchical [22] and requires specific associations between AZ proteins [23].

Nearly all CAZ proteins identified at invertebrate synapses are also present at mammalian synapses, suggesting a significant conservation of function throughout evolution [23] (Fig 2). Two exceptions are Piccolo and Bassoon, which may have evolved to perform vertebrate-specific functions. However, studies by Bruckner *et al* [24] have recently identified a Piccolo homolog, aptly named Fife, which is selectively localized at *Drosophila* AZs. Structurally, Fife is a hybrid molecule with features from both Piccolo and RIM,

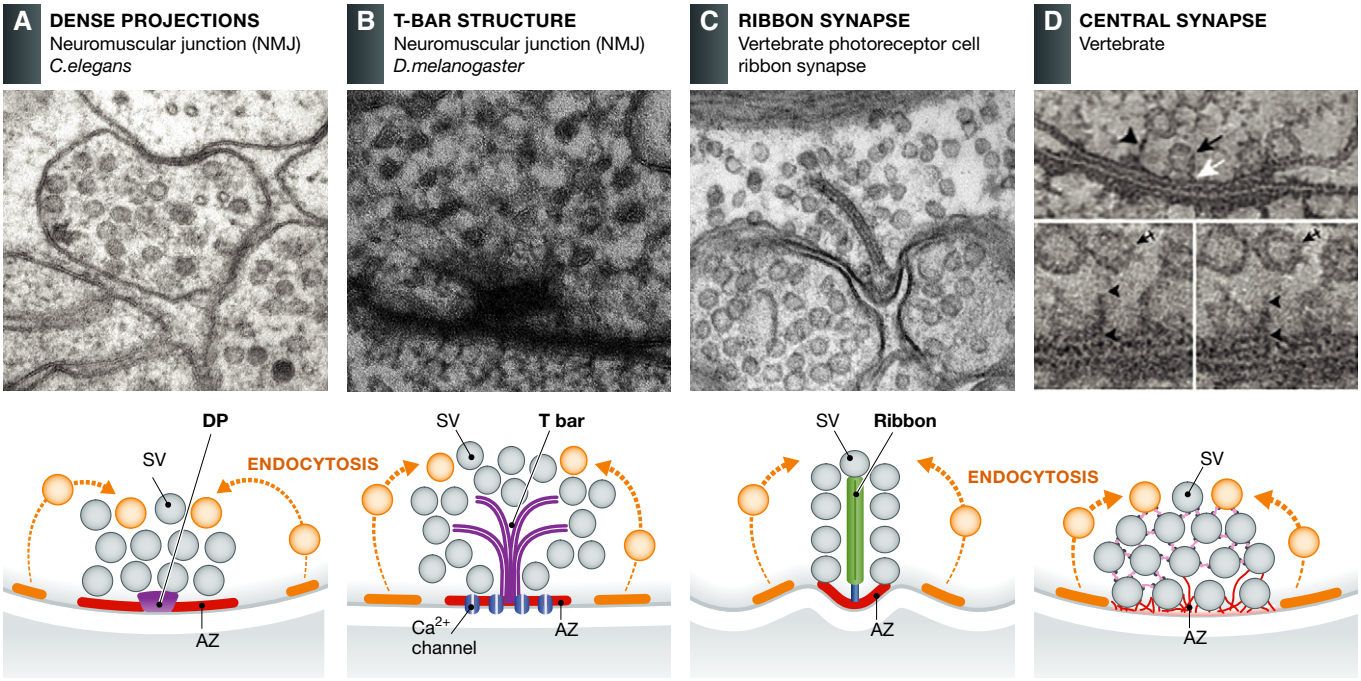

Figure 1. Active zones display different morphologies.
Electron micrographs and schematic drawings reveal that AZs can be divided into morphologically distinct groups: those with elaborate electron-dense projections such as T-bars and ribbons and those with less prominent dense projections including *C. elegans* active zones and those present at most vertebrate central nervous system synapses. (A) AZ from a *C. elegans* NMJ. In general, these AZs are quite simple and generally characterized as a broad surface of plasma membrane situated between electron-dense projections (DPs) and flanking cellular tight junctions. (B) AZs present at the NMJ of the fly *Drosophila melanogaster*. These are more elaborate forming a platform consisting of a meshwork of filaments overlaying a pedestal which gave them the name T-bars. (C) Vertebrate photoreceptor cell ribbon synapse. These synapses are characterized by a large AZ with a specialized organelle, the synaptic ribbon, which tethers large numbers of SVs near the AZ, facilitating fast-sustained synaptic transmission. (D) AZs of vertebrate central synapses are less complex than at sensory synapses, exhibiting fine filamentous projections that connect proximal (docked vesicles) and more distally located SVs, up to 100 nm, to the plasma membrane holding them close to the release sites. In schematic drawing, active and endocytic (not shown in EM micrograph) zones are marked as red and orange, respectively. Reproduced with permission, from [4] (A); Hollmann and Sigrist (B); [155] (C); [11] (D).

Table 1. Active zone proteins in invertebrates and vertebrates.

Protein	Function	References
C. elegans		
SYD-2/Liprin-α	Synaptic scaffolding protein: active zone morphology; SV docking; synaptic transmission	[8,9]
UNC10	Synaptic vesicle priming factor: SV docking,SV priming, calcium channel localization	[10,19]
Drosophila		
Bruchpilot	Synaptic scaffolding protein: component of the T-bar structure; calcium channel clustering	[15–17]
dRBP	Synaptic scaffolding protein: calcium channel clustering	[20]
Fife	Synaptic scaffolding protein: active zone organization	[24]
Neurexin	Synaptic adhesion protein: synapse assembly, synapse growth	[26]
Neuroligin	Synaptic adhesion protein: synapse assembly	[25,27]
Intersectin	Endocytic protein: SV fusion, membrane retrieval	
Mice		
RIM	Synaptic vesicle priming factor: SV docking, SV priming, calcium channel localization	[60,61,68–70]
Munc13	Synaptic vesicle priming factor: SV priming, SV fusion	[66,67,70]
RBP	Synaptic scaffolding protein: calcium channel clustering	[20]
Bassoon	Synaptic scaffolding protein: ribbon attachment, calcium channel clustering, synapse integrity	[46,48,51,151]
Piccolo	Synaptic scaffolding protein: actin assembly, synapse integrity	[119–121,151]
Piccolino	Synaptic scaffolding protein: ribbon shape	[52]

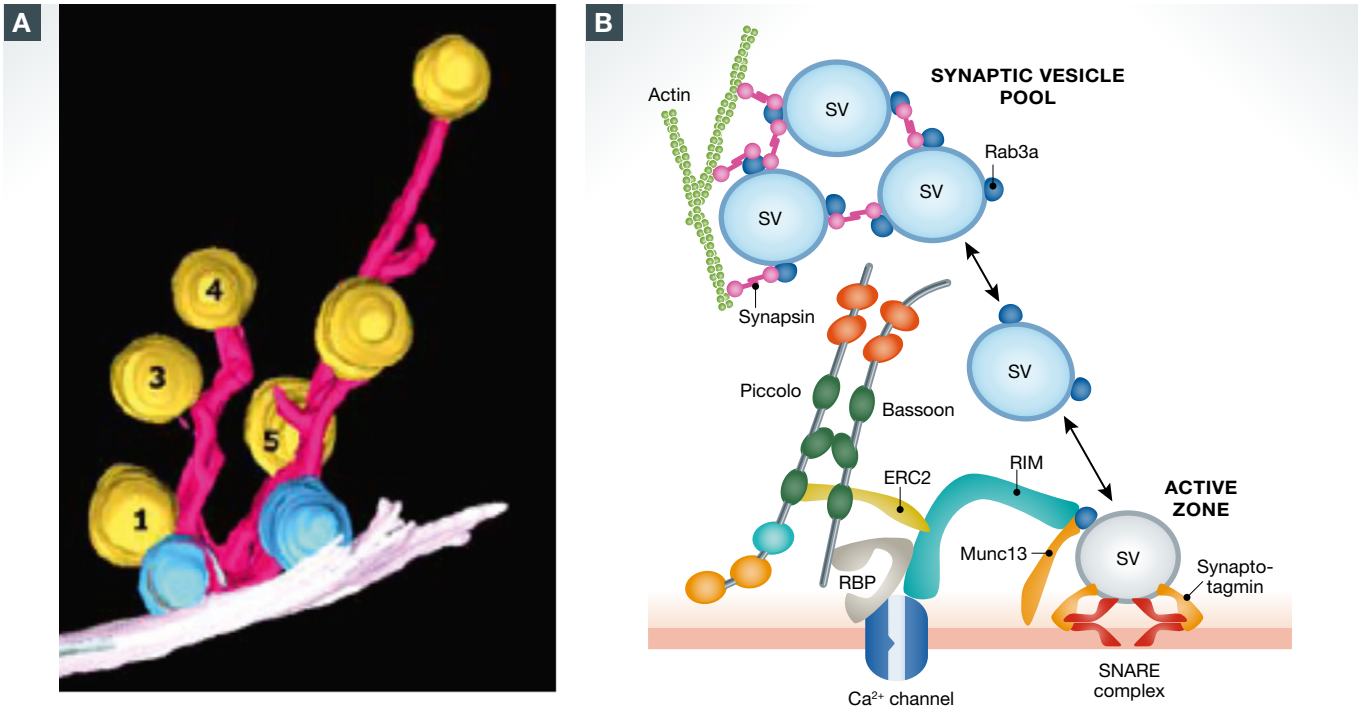

Figure 2. Cytoskeletal matrix proteins organize synaptic vesicle release sites at presynaptic active zones.
(A) 3D reconstruction of filaments (pink) and SVs (yellow) tethered near the active zone from a vertebrate (rat) hippocampal synapse. Docked vesicles are in blue. Reproduced with permission from [11]. (B) Schematic diagram of CAZ molecules mediating the capture (synapsin, actin), docking (SNARE complex), priming (RIM, Munc13, Rab3) and fusion (synaptotagmin) of SVs and VGCCs (Bassoon, RBP, RIM, ELKS) at presynaptic active zones. At present, the spatial relationship of these molecules within the cryo-fixed EM image in (A) is not well resolved.

including zinc finger (ZnF), PDZ, and C2 domains. Fife loss-of-function has profound effects on AZ organization and synaptic transmission, including a dramatic decrease in excitatory junctional potential amplitude and quantal content. Morphologically, boutons lacking Fife exhibit a 20% reduction in the number of SVs associated with T-bars, as well as floating T-bars and detachment of pre/postsynaptic membranes. Intriguingly, floating T-bars are occasionally seen at fly NMJs lacking RBP [21], suggesting that Fife and RBP both function to tether SVs at the AZ. Furthermore, the detached membrane phenotype implies a role in transsynaptic adhesion [24].

Interestingly, several CAZ proteins have been identified to work in concert with the transsynaptic adhesion/signaling molecules Neurexin-1 (Nrx-1) and Neuroligin-1 (Nlg1) at the *Drosophila* NMJ, including Syd1, Syd2/DLiprinα, and Wrd (a regulatory subunit B' of the phosphatase PP2A). As at mammalian synapses, Nrx-1 and Nlg1 facilitate synapse assembly at the *Drosophila* NMJ, and their loss-of-function causes severe synaptic assembly defects [25–27]. Mechanistically, Nrx-1 appears to promote synapse assembly by directly interacting with presynaptic Syd-1. Syd-1 in turn interacts with and stabilizes nascent Syd2/DLiprin-α clusters critical for early synapse assembly [26] and BRP recruitment [28], linking transsynaptic adhesion and active zone scaffold assembly. Of note, the stabilization of these transsynaptic complexes and the inhibition of ectopic synapse assembly also require Wrd/PP2A through its association with DLiprin-α [29]. At present, it is unclear whether this same ensemble of presynaptic proteins promotes synapse formation at vertebrate synapses discussed below. Another important question is how activity regulates the remodeling of AZs, and whether this process is fundamentally different from normal development.

Vertebrate active zones

Vertebrate AZs share many of the same features and core proteins of invertebrate synapses. One major difference is the molecular diversity of CAZ proteins, which arise from both gene duplications and alternative splicing. Why is so much diversity necessary? A likely answer is that molecular diversity allows vertebrates to build more elaborate AZs with greater functional diversity, permitting more nuanced modulation of synaptic responsiveness and plasticity. In exploring this concept, we will first discuss how the assembly of unitary release sites contributes to morphologically distinct AZs present at sensory and central synapses. We will then describe emerging roles of AZ proteins that have been uncovered in recent studies, which include coupling SV exocytosis with endocytosis, and regulating synapse plasticity and integrity. Open questions in these areas are highlighted in Sidebar A.

CNS synapses

The organization of AZs at vertebrate central nervous system (CNS) synapses resembles that seen in invertebrates. For instance, core AZ proteins involved in the regulated release of neurotransmitters (i.e., RIMs, Munc13, RBP, SNAREs, complexin, synaptotagmin, and Munc18) are also present at vertebrate synapses. Morphologically,

Sidebar A: In need of answers

Tremendous progress has been made in the molecular and functional characterization of proteins that define presynaptic AZs. These analyses have revealed significant functional and structural conservation across species, allowing information garnered from studies of the invertebrate AZ to be applied to vertebrates and vice versa. However, many outstanding questions remain. For example

- Why are there so many additional isoforms for each AZ protein in vertebrates? Do they simply allow for functional diversity, or also for structural diversity? In order to answer this question, we will need a better understanding of the nano-domain organization of AZs.
- How are active and peri-active/endocytic zones created and maintained?
- Do presynaptic AZs contain functionally distinct micro-domains that support specific types of neurotransmitter release (i.e., spontaneous versus phasic release)? One attractive but untested idea is that transsynaptic adhesion molecules define these nano-domains by triggering the bidirectional assembly of subsets of pre- and postsynaptic proteins.
- What regulates the integrity of AZs? Recent studies have uncovered several exciting and unanticipated functions of CAZ proteins, such as regulating protein homeostasis mechanisms critical for synaptic health and integrity. It will be important to address whether and how the CAZ regulates the formation and trafficking of autophagic and endocytic structures. Clearly, this topic has fundamental importance for our understanding of neurodegenerative disease mechanisms.
- What mechanisms regulate fast synaptic transmission and plasticity? Live imaging studies reveal that AZs are highly dynamic structures that undergo continuous remodeling and turnover. However, our current understanding of the AZ is based primarily upon static images and the dynamics of just a few CAZ molecules, and is therefore unable to give us a complete picture of the molecular interactions that underlie fast synaptic signaling and plasticity. Thus, it is imperative for future studies to investigate the dynamics of CAZ proteins at high temporal and spatial resolution.

AZs at CNS synapses are far less complex than those observed at sensory synapses discussed below. Initial studies using conventional EM revealed that AZs in the CNS were organized into pyramidal-shaped structures interconnected by 50- to 100-nm-spaced fibrils, forming slots for synaptic vesicles to dock and fuse [1,30]. However, in cryo-fixed material examined by electron tomography [11], such lattices were not detected. Instead, numerous fine filaments were observed to project from the surface of the AZ, contacting docked synaptic vesicles and those up to ~100 nm from the plasma membrane (Figs 1–3) [8,11]. These findings suggest that the pattern seen in aldehyde-fixed material may be an artifact of this fixation process, caused by protein deposition between docked synaptic vesicles. Intriguingly, the filamentous structures emanating from the AZ in these cryo-fixed preparations resemble those observed at invertebrate synapses (Figs 1 and 2), suggesting that the molecular structures responsible for capturing and guiding SVs to their fusion sites are evolutionarily conserved. These images also indicate a role for CAZ proteins beyond the tethering of calcium channels or the priming of SVs (e.g., RBP, Munc13, and RIMs), a concept that will be discussed more fully below in the context of lessons learned at vertebrate NMJs.

A major challenge facing the field is how to assign molecular identities to the filaments and structures observed in these images. Strategies being employed to resolve this issue include the use of super-resolution and immuno-electron microscopy. Super-resolution microscopy is emerging as a powerful approach to understand the spatial relationships between different presynaptic molecules, as well as the number of such molecules present within boutons [31–33]. Of particular note is a visually stunning study performed by Wilhelm *et al* [33] designed to define the number and relative distributions of presynaptic molecules per bouton (Fig 4). Such images nicely complement proteomic-based studies [34], and challenge the community to look beyond our favorite molecules as we try to understand the intricacies of synapse architecture and function. However, it is important to note that such studies also have their limitations. For example, many of the molecules associated with AZs are very large (> 100 kDa), complicating the assignment of their localization based on immunostaining with a single antibody. Furthermore, the information derived from these experiments is relative, for example, based on the distance to one or two other molecules co-labeled in a given experiment. Such limitations argue for caution in the acceptance of models based solely upon such data.

A complementary approach, providing information about the molecular composition of the AZ, is immunogold labeling of cryo-fixed material combined with electron tomography. Siksou and colleagues have used this strategy to show that the SV-associated protein Synapsin is one component of the short filaments between SVs within the reserve pool. Their data also indicate that the longer, AZ-attached filaments might represent Bassoon tethered to the plasma membrane-associated AZ protein CAST/ERC [11]. More recent immuno-EM studies indicate that RIM1α is a component of the fine filaments tethering SVs to the AZ plasma membrane [35]. One downside of this approach is the technically demanding nature of experiments, highlighting the need for innovation in order to accelerate data acquisition in the realm of EM tomography.

Neuromuscular junctions

Two major limitations of CNS synapses with respect to AZ structure are their small size and their intrinsic variability. This was illustrated in studies by Schikorski and Stevens [36], who showed that the number and geometric relationship of release sites per bouton were highly variable in CNS synapses, a situation that hampers the assignment of molecules to specific filaments across AZs. Fortunately for synaptic biologists, nature created the neuromuscular junction (NMJ), a synapse formed between motor neurons in the ventral horn of the spinal cord and individual muscle fibers. AZs at this synapse are very long (~1 μm) and have a very regular, stereotyped pattern. Detailed ultrastructural studies reveal that SVs docked at the NMJ are tethered to the AZ by four classes of macromolecular structures: ribs, pins, spars, and booms (Fig 3) [37,38]. More recently, these dense projections have been further divided into three sublayers, each defined by specific electron-dense macromolecules [3]. The superficial layer, adjacent to the presynaptic membrane, consists of beams, ribs, and pegs; it is followed by the intermediate layer, defined by steps and spars, and finally the deepest layer, containing masts, booms, and topmasts (Fig 3) [3].

These elegant findings will revolutionize our understanding of how CAZ proteins facilitate the efficient docking and fusion of SVs. For instance, one is struck by the regularity with which the mast,

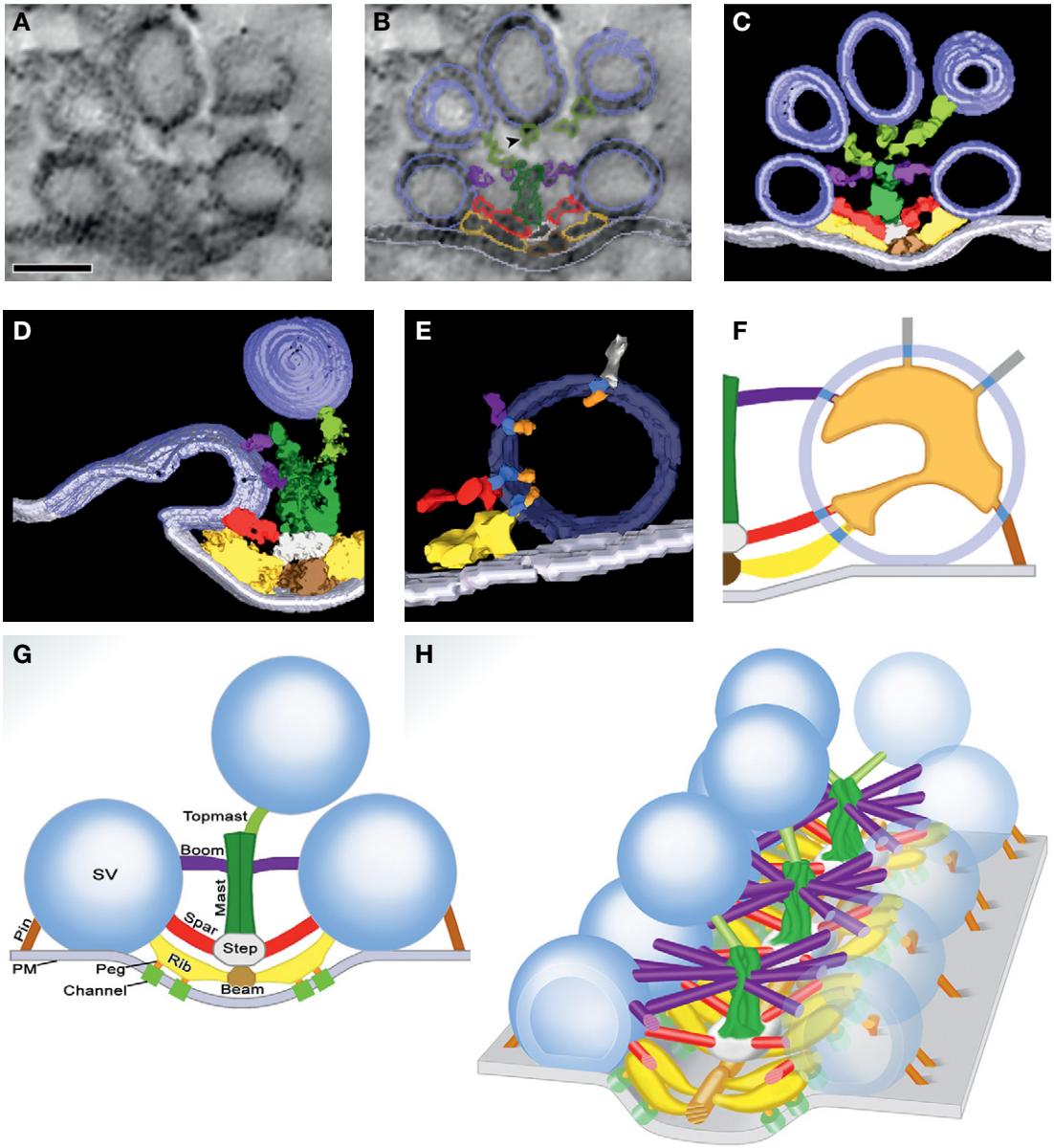

Figure 3. Macromolecular organization of the frog NMJ.
At the NMJ of the frog, SVs are arranged in highly organized linear arrays. The precise alignment of the vesicles is achieved through different filamentous structures, which contact the vesicles. (A) Electron micrograph showing subdomain organization of SVs at the frog NMJ. Synaptic vesicles are held in place through filamentous structures. Scale bar, 50 nm. (B) Schematic representation of the electron micrograph in (A) with summed outlined of AZ material (AZM) macromolecules colored. (C) 10-nm-thick surface model of AZM and SVs shown in (A) and (B), derived from eight adjacent slices. Docked vesicles close to the plasma membrane are attached to several filaments contrary to vesicles further away that are only connected to one filament. (D) 25-nm-thick surface model of an AZ, capturing a former docked vesicle that has fused with the plasma membrane but remains in contact with ribs, spars, and boom implying that AZM participates in SV fusion. (E) Surface model, ~10 nm thick, showing in 3D the nubs linked by transmembrane bands connected to rib, booms, and spars. (F) Schematic diagram of the luminal assembly of macromolecules within a docked SV that form contact with rib, boom, spar, and pin as well as non-AZM molecules. (G) Composition diagram of layers of AZM shown in transverse plane of the AZ at frog NMJ. The main body of AZM includes beams, ribs, and pegs; the intermediate layer: steps and spars; and the deep layer masts, booms, and topmasts. (H) Diagram of NMJ revealing the repetitive array of core AZ proteins centered around a mast (green) that contacts surrounding SV through ribs, spars booms and topmasts. This geometric arrangement allows the creation of tens of SV docking and fusion sites. Reproduced with permission from Szule and Harlow [3,39].

boom, and ribs are assembled. From each mast core, the booms, spars, and topmast extend radially to contact 4–5 SVs. This arrangement indicates that AZs of the NMJ are assembled by the sequential addition of these structures, creating a linear array of AZs. Intriguingly, these structures are highly reminiscent of the radial AZs created by BRP at the *Drosophila* NMJ (Fig 1), implying a conservation of AZ core structures across evolution.

A second concept addressed by these morphometric studies is the function of filaments emanating from the AZ. On average, each SV docked at the AZ is connected to four ribs, four pins, two spars,

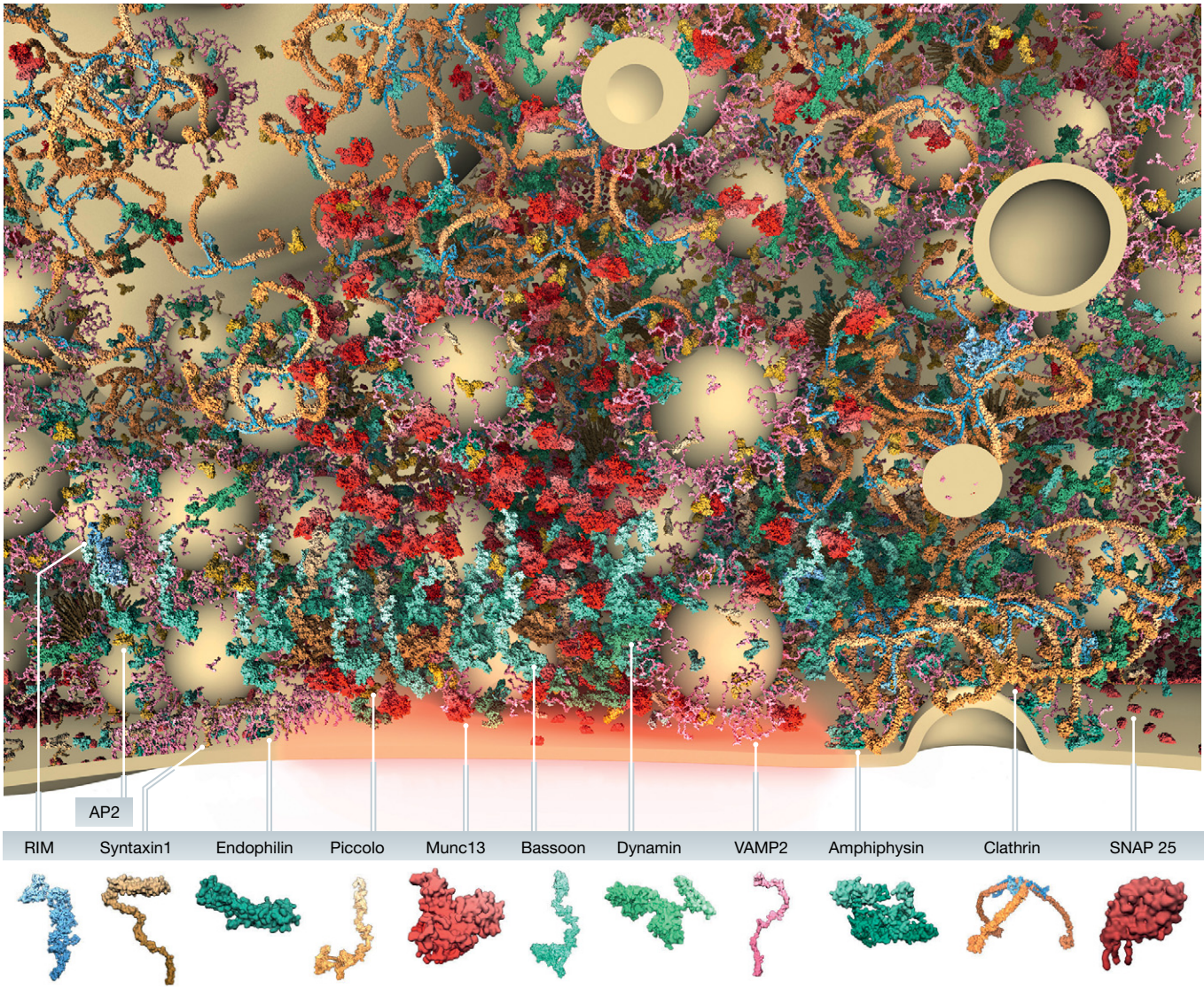

Figure 4. Model of presynaptic bouton and active zone organization.
A section through the active and endocytic zones of a vertebrate synapse indicating the spatial distribution and copy number of presynaptic proteins that help define the presynaptic AZ as the site of SV exocytosis and clathrin-mediated endocytosis. Panel at the bottom, a graphical legend of the predicted structures of presynaptic proteins included in the model. Displayed SVs have a diameter of 42 nm. The image was generously created by Burkhard Rammner in the Rizzoli Laboratory. See also [33].

and five booms (Fig 3) [3]. With each SV fusion step, SVs in the reserve pool replace docked SVs, forming contact with each of the four classes of CAZ molecules. Intriguingly, the number of contacts is inversely proportional to SV distance from the plasma membrane, suggesting that these filaments guide SVs to their docking sites, helping to position them near VGCCs.

At present, the identity of the proteins that define each class of filaments remains unclear. Yet given the almost crystalline arrangement of the masts, booms, and spars, one can see how this preparation is ideal for defining their composition and generating hypotheses regarding their functions. For example, given the proximity of ribs to the plasma membrane and their association with VGCCs, one could infer that they comprise proteins such as RIM, Munc13, and RBP, which are involved in SV priming and VGCC tethering. Consistent with this idea, these filaments remain associated with the SV membrane during fusion [3].

It is worth mentioning a third provocative observation arising from these studies. Specifically, using freeze substitution methods, Harlow *et al* [39] have found that the lumen of SVs is not only filled with neurotransmitter, but also contains an assembly of macromolecules organized into a shape that exhibits chirality and is similar from vesicle to vesicle. Moreover, this structure has arms that radiate from the center of each vesicle and connect via nubs to the surface of SVs (Fig 3). These in turn contact the ribs, booms, spars, and pegs of the CAZ. Importantly, the relative orientation of this chiral SV assembly toward the CAZ is identical for all docked SVs (Fig 3). This intriguing observation indicates that the CAZ plays an active role in orienting each SV with respect to the plasma

membrane. It also suggests that the organization of SV proteins is not random and that AZs use these tethers to position proteins involved in fusion (i.e., VAMP-2, synaptotagmin-1) toward the plasma membrane. It will be interesting to see whether this chirality is preserved at other CNS synapses, and how its disruption alters SV release kinetics.

Ribbon synapses

Although AZs of the vertebrate NMJ are complex, sensory synapses of the retina and auditory system have taken AZ design to a yet higher level. Similar to the NMJ, AZs of retinal ribbon synapses are elongated structures, called "archiform densities", with a row of docked SVs flanking the central axis. Attached to each archiform density is a specialized organelle, the synaptic ribbon, that is perpendicular to the plasma membrane (Fig 1) and tethers large numbers of SVs near the AZ, an arrangement that facilitates rapid, sustained synaptic transmission [40,41]. At present, the principles that organize the assembly of ribbon synapses are not known, though it is provocative to consider that ribbon synapses, like the NMJ, employ specific classes of scaffold proteins to (i) build the ribbon and (ii) tether it along a longitudinal array of AZs. Consistent with this concept, the molecular characterization of retinal ribbon synapse reveals that each compartment is defined by distinct groups or subfamilies of CAZ proteins. For example, major structural components of photoreceptor and bipolar cell ribbons include Ribeye, CtBP1, Piccolo, Kif3A, and RIM1 [41–44]. In contrast, the archiform density is comprised of proteins involved in synaptic vesicle fusion and calcium channel clustering (RIM2, ubMunc13-2, Liprins, CASTs/ERCs/ELKS, Bassoon) [41]. Loss-of-function studies have identified several of these that dramatically affect the integrity of ribbon synapses. The most remarkable is the multifunctional CAZ protein Bassoon, which is physically anchored to the archiform density [45]. In photoreceptor and hair cells of Bassoon mutant mice, ribbons are no longer attached to AZs and float freely in the cytoplasm [46–48], implying a crucial role for Bassoon in tethering ribbons to the archiform density. Loss of Bassoon also reduces the number of synaptic Ca^{2+} channels tethered at SV release sites [48]. While less dramatic, the inactivation of CAST/ERC2 (a Bassoon/RIM binding partner) [49,50] leads to a concomitant decrease in AZ size and complement of VGCCs, without affecting the attachment of ribbons, implying a role for CAST/ERC2 in AZ organization [51]. A third critical component of ribbons is a short isoform of Piccolo, Piccolino, which is predominantly expressed at sensory ribbon synapses of the eye and ear [44]. At these sites, Piccolino loss-of-function is associated with a dramatic change in ribbon shape, leading to the presence of spherical rather than plate-shaped ribbons [52]. Intriguingly, during ribbon biogenesis, precursor spheres are associated with the initial assembly of ribbons, maturing from spherical to plate-shaped ribbons during light adaptation [53,54]. This transition is disrupted in the absence of Piccolino, implying a role for this scaffold protein in the formation of plate-shaped ribbons [52]. This function is complementary to that performed by Ribeye, which promotes the formation of spheres [55], and to Bassoon, which anchors them to the archiform density [54].

Further progress in this field will require a better understanding of the molecular organization of these synapses and the dynamics of their assembly. Equally important will be the characterization of other sensory synapses such as those found in the cochlea [56], where intriguing similarities and differences are being uncovered. For example, while hair cell ribbons require Bassoon for their attachment, they use Otoferlin rather than Munc13 for SV exocytosis [57–59]. Finally, it will be important to better understand the macromolecular principle of assembly and whether rules defined at simpler synapses are utilized wholly or in part at these highly specialized synapses.

Active zone and release probability

The reliability of synaptic transmission is dependent on many factors. Among the most important are the efficiency of SV docking and priming, and the proximity of Ca^{2+} channels to SV release sites. Several CAZ proteins are key regulators of these events. For example, RIM1 is essential for SV priming [60,61], and also regulates Ca^{2+}-channel localization by directly binding to the C-terminal tails of N- and P/Q-type Ca^{2+} channels via its PDZ domain [61]. Conditional RIM1/2 knockout mice exhibit a reduction in presynaptic Ca^{2+}-channel density at SV release sites [62]. A similar phenotype is seen in *Drosophila*, where RIM1 loss-of-function also leads to a reduced density of Ca^{2+} channels at NMJs [19]. However, RIM is not the only AZ protein responsible for the subcellular localization of Ca^{2+} channels. For example, Bassoon loss-of-function also reduces the density of channel subunits ($Ca_v2.1$) at the AZ, through an interaction with RIM-binding protein (RBP) [63]. A central role for RBP in calcium channel clusters has also been shown in *Drosophila* [21].

Synaptic vesicle priming, a process that activates SVs for exocytosis, is another important determinant of vesicular release probability (for a comprehensive review, see [64]). Two AZ proteins critical for priming are RIM and Munc13. Munc13 plays an essential role in converting the SNARE complex protein Syntaxin-1 into an open conformation, allowing for formation of the full SNARE complex with VAMP-2 and SNAP25, and thus SV fusion [65–67]. In contrast, RIMs are thought to play a structural role during SV priming, recruiting Munc13 to the AZ (through an interaction between their N-terminal Zn^{2+}-finger (Znf) domains and the N-terminal C_2A domain of Munc13) [68,69]. However, more recently, this interaction was shown to functionally convert Munc13 from an inactive homodimer into an active RIM/Munc13 heterodimer, thereby activating its priming function [70]. Consistent with these findings, studies in *C. elegans* show that interactions between RIM/Unc10 and Unc13 are important not only for efficient neurotransmitter release, but also for the precise localization of Unc13 within the AZ [71]. For instance, in Unc13 mutants lacking their RIM-binding C_2A domains, SV docking occurred distal to the AZ, potentially displacing SVs from their calcium source and thus reducing release probability [71]. In addition, Unc13 paralogs and isoforms may contribute functionally to synaptic heterogeneity at vertebrate synapses. For example, while all Unc13 isoforms share a similar C-terminus, they differ significantly in their N-termini. On one hand, some isoforms lack the C2A domain important for RIM interaction, leading to less efficient and more asynchronous release in *C. elegans* [72]. Even among the mammalian Munc13 isoforms (Munc13-1 and ubMunc13-2), both of which contain functional C2A domains, release probability and short-term plasticity characteristics differ significantly [73,74], indicating that Munc13 regulates release efficiency through several mechanisms.

Importantly, changes in SV release probability, and thus synaptic reliability, can be dynamically regulated. In general, synaptic strength/efficacy correlates with the number of readily releasable vesicles (N) docked at the AZ, which are proportional to the active zone area and their vesicular release probability (Pvr) [75,76]. Thus, larger terminals typically have higher synaptic release probabilities [77,78]. Changes in these parameters during synaptic plasticity can alter neurotransmitter release. For example, the size and number of active zones in Aplysia, as well as the number of vesicles close to the plasma membrane, change upon habituation or sensitization of the gill-withdrawal reflex [79]. Similarly, facilitated NMJ synapses in the crayfish have more AZs and an increased number of readily releasable SVs [80]. These observations imply a tight relationship between the functional properties of a synapse and the size of the AZ.

Recent studies have begun to elucidate the molecular underpinnings of this relationship. For example, at the *Drosophila* NMJ, changes in vesicular release probability and readily releasable vesicle pool size during synaptic plasticity correlate with AZ size, as well as with levels of the core AZ protein BRP [81]. Intriguingly, these structural changes can be fast, occurring within minutes after plasticity induction, as well as long-lasting [81]. Importantly, conditions that promote homeostatic changes in synaptic strength and release probability trigger changes not only in the composition of CAZ proteins, but also in key regulators of vesicular release probability such as synaptotagmin and P/Q-type calcium channels [82]. Studies in *Drosophilia* suggest that one key regulator in this process is the AZ protein RIM [83].

These observations raise fundamental questions about how synaptic activity sculpts AZ size and vesicular release probability. One emerging theme is that bidirectional signaling, via a growing number of transsynaptic cell adhesion molecules [84–86], couples changes in postsynaptic responsiveness with presynaptic efficacy. The best-characterized of these is the Neurexin–Neuroligin (Nrx/Nlg) complex, whose postsynaptic activation can potently enhance presynaptic size and reliability [87,88], through a still-unknown mechanism. Studies in *Drosophila* suggest that Syd1 is a critical molecule in this signaling cascade, as its binding to the C-terminal tail of Nrx1 controls not only the number and stability of Nrx1/Nlg1 complexes, but also synaptic size and reliability [26].

A second emerging theme is bidirectional signaling between the presynaptic compartment and the nucleus. While transsynaptic signaling can promote real-time changes in synaptic strength, long-lasting changes and homeostatic plasticity are known to require changes in gene expression. The best-characterized mechanism involves the translocation of signaling molecules from the postsynaptic density to the nucleus [89]. Studies by Ivanova and colleagues recently found that presynaptic AZs also participate in this conversation. Specifically, the Bassoon binding partner CtBP1 is a transcription factor that transits between presynaptic AZs and the nucleus in response to homeostatic changes in synaptic strength [90]. How CtBP1-mediated signaling regulates synaptic size and function remains to be explored.

Exo-endocytosis coupling

After priming, readily releasable SVs fuse with the plasma membrane and release neurotransmitter upon Ca^{2+} influx. To maintain exocytosis, release sites must not only be cleared of excess synaptic proteins and membranes, but these proteins must also be retrieved and reassembled into functional SVs, necessitating tight coupling between exocytosis and endocytosis [91,92].

Emerging evidence points toward a role for CAZ scaffold proteins in this coupling, though many questions remain. Over the last decade, most efforts have focused on whether and how exo- and endocytosis are coupled. Relevant to this issue is the type of endocytosis being examined. Until recently, three forms of endocytosis have been described at synapses: kiss-and-run, clathrin-mediated, and bulk [93] (Fig 5). Kiss-and-run, in which vesicles do not fully fuse with the membrane but transiently flicker open at a fusion pore, is the fastest of these, occurring within ~1 s [94,95]. Clathrin-mediated endocytosis (CME) is the most common form of endocytosis [96], facilitating retrieval of SV proteins in tens of seconds following full-collapse fusion of SVs. CME occurs in a spatially separate region of presynaptic plasma membrane referred to as the peri-active zone [93,97] (Fig 5). Bulk endocytosis is a pathway triggered by strong stimulation, mediating the retrieval of SV proteins via large endocytic vacuoles. Clathrin-mediated budding is subsequently used to regenerate new SVs from these structures [98,99] (Fig 5).

Unfortunately, all three mechanisms are rather slow, making it difficult to explain how sufficient SV regeneration can occur at high firing rates. In a groundbreaking set of studies, Watanabe *et al* have used a "flash-freeze" approach to capture an "ultrafast" form of endocytosis that occurs within 50 and 100 ms after stimulation [100,101] (Fig 5). Importantly, these authors find that ultrafast endocytosis captures ~ four vesicles at a time and occurs at the very edge of the AZ, implying that endocytic zones are functionally and perhaps physically linked to AZs. However, this linking mechanism remains unknown.

One obvious link between the AZ and endocytic zone is calcium, which plays fundamental roles in exocytosis [102]. Studies at the frog NMJ provided early evidence that endocytosis requires calcium. Here, α-Latrotoxin, a spider toxin that triggers calcium-independent exocytosis of SVs, was used to show that low levels of extracellular calcium depress endocytosis following α-Latrotoxin-mediated SV exocytosis [103]. This conclusion is also supported by calcium buffers such as BAPTA that decreases endocytosis rates at synapses by up to 1,000-fold [104]. Consistently, calcium itself has been found to regulate endocytosis at synapses of different types [105–109].

A fundamental but unresolved question is where this calcium comes from. One concept, put forth by Yao and colleagues, is that SVs carry VGCCs, which are inserted into the plasma membrane following SV exocytosis, supplying the calcium necessary for endocytosis. One potential candidate, identified in *Drosophila*, is the vesicular protein Flower [110]. However, while Flower appears to be a calcium channel, its conductance is small [110]. Moreover, subsequent studies failed to find evidence that exocytosis transiently inserts calcium channels into the plasma membrane [111]. Rather, these authors found that more traditional VGCCs (e.g., P/Q-type) provided the calcium essential to trigger both rapid and slow endocytosis at the presynaptic membrane of the Calyx of Held [111] (see also [92]). As discussed above, several of these VGCCs are physically tethered to AZs by the scaffold protein RIM and RBP [17,21,61], allowing rapid coupling between action potential-mediated calcium influx and SV fusion. Yet as discussed below,

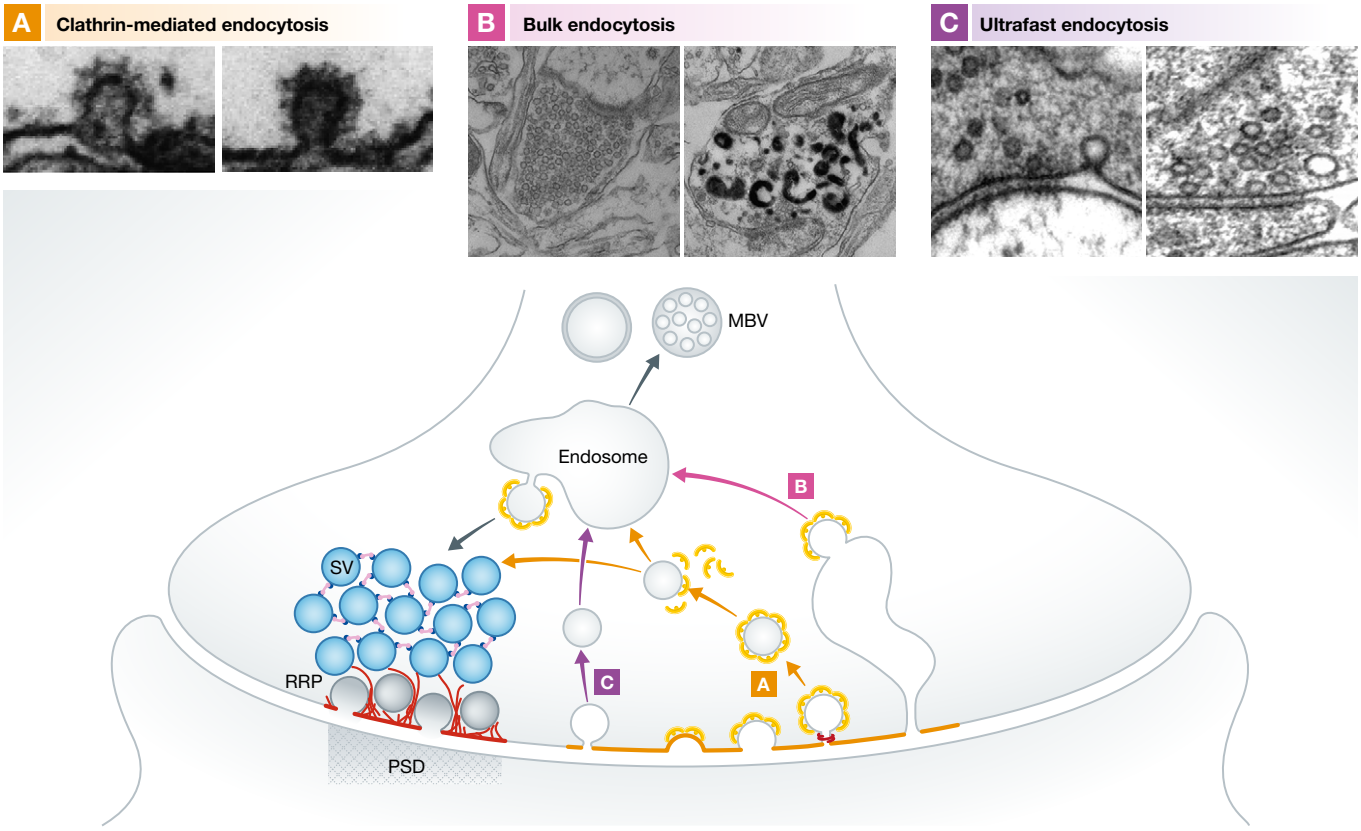

Figure 5. Synaptic vesicle cycle at the presynaptic terminal.
The presynaptic AZ functionally defines the space within boutons where upon calcium influx synaptic vesicle fusion and neurotransmission takes place (lower panel). It is the center of the SV life cycle. Vesicles are recruited from the vesicle cluster toward the AZ where they undergo maturation steps such as docking and priming and finally fuse with the plasma membrane upon action potential stimulation. After exocytosis, SV protein and membrane retrieval occurs through endocytosis in a region spatially adjacent to the active zone, the peri-active zone. It is an important compensatory reaction to recapture excess membrane and generate new SVs. Different endocytosis pathways are known and can be visualized by EM following evoke stimulation (top panels). Clathrin-mediated endocytosis (A) is a slow form of membrane retrieval. Synaptic vesicle proteins and membrane are taken up through the formation of clathrin-coated pits, which are later pinched off from the membrane through the GTPase dynamin. After uncoating, the newly formed vesicles either join the vesicle cluster or pass an additional sorting step through an early endosome. Bulk endocytosis (B) is a second form of endocytosis that mainly takes place during strong stimulation. Large membrane fractions are collected in big invaginations and pinched off from the plasma membrane. New vesicles are then formed via clathrin-mediated budding from these structures. Most recently, a new mode of endocytosis has been described, ultrafast endocytosis (C). It is a very fast retrieval mechanism as it can take place within 50–100 ms after stimulation. Vesicles are pinched off from the plasma membrane at the edge of the active zone. Free vesicles fuse with a sorting endosome from which new synaptic vesicles are formed in a clathrin-dependent manner. Top panels, EM micrographs of clathrin-mediated (A), bulk (B), and ultrafast (C) endocytosis. Reproduced with permission from Frauke Ackermann, Joshua A. Gregory, and Lennart Brodin (A); [156] (B); [100] (C).

endocytic zones lie in a peri-active zonal region [93], raising questions of whether calcium entry at the AZ remains at sufficiently high levels to facilitate endocytosis [see also 92,112]. Intriguingly, endocytosis still occurs at the *Drosophila* NMJ when the main AZ VGCC, cacophony, is absent [113], but can be inhibited by several different calcium channel blockers. These data suggest that exocytosis and endocytosis may use distinct calcium channels [113,114; see also 91], a concept supported by experiments at the calyx of Held [115], but raise new questions of how these channels are tethered near AZs.

Studies at both *Drosophila* NMJs and rat hippocampal synapses are beginning to provide answers to these questions. For example, super-resolution microscopy was recently used to show that several different endocytic proteins, for example, Intersectin, Stonin, and GIT1, exhibit a punctate peri-active zone-like pattern around T-bars and AZs labeled with BRP or Bassoon, respectively [116–118]. These findings suggest that SV exocytosis sites are surrounded by an array of discrete endocytic zones or hot spots. This radial arrangement implies the presence of molecular tethers that link them to AZs.

At present, the identities of these tethers are unknown. Potential candidates include the AZ scaffold proteins Piccolo, Bassoon, ELKs/CAST, or RIM, as their large sizes could bridge multiple presynaptic micro-domains over distances of several hundred nanometers. Of these, Piccolo is the most intriguing, as it has been found to scaffold proteins critical for endocytosis. These include Profilin and Daam1, which together with Piccolo promote F-actin assembly within presynaptic boutons in an activity-dependent manner [119,120]. Piccolo also binds Abp1 and GIT1 [121,122]. The former is an actin-binding protein that also interacts with dynamin, another key

endocytic protein, while the latter is a G-protein-coupled receptor kinase-interacting (GIT) protein that directly associates with the endocytic adaptor protein Stonin2/StonedB [118]. In synapses lacking GIT1, Stonin2/StonedB is displaced and SV recycling impaired, implying that GIT1 acts as a bridge between endocytic zones, defined by Stonin2/StonedB, and active zones [118]. Whether the Piccolo-GIT1 association [122] is a further component of this bridge will require further investigation. Intriguingly, the dynamic assembly of F-actin is required for ultrafast endocytosis and bulk endocytosis [100,123,124]. Given the capacity of Piccolo to act as a platform for F-actin assembly during activity-induced SV recycling [120], it will be important to explore its involvement in these endocytic events.

Intersectin is another up-and-coming candidate for spatial coupling of the exocytic and endocytic machinery. It is a multi-domain endocytic protein existing in two vertebrate isoforms ITSN-S and ITSN-L [125]. ITSN-S consists of two N-terminal EH domains, a coiled-coil region, and four or five SH3 domains. The longer isoform ITSN-L also possesses C-terminal DH, PH and C2 domains [126] providing it with guanine nucleotide exchange factor (GEF) properties for Cdc42 [127]. Intersectin interacts with several endocytic proteins including dynamin, AP2, epsins, and synaptojanin. Loss-of-function studies in *C. elegans* and *Drosophila* show mislocalization of dynamin, endophilin, and synaptojanin, as well as impaired endocytosis [128,129]. In vertebrates, intersectin is important not only for endocytosis, but also for exocytosis [130], as ITSN1 loss-of-function impairs exocytosis in PC12 cells and chromaffin cells as well as at the Calyx of Held [116,131,132]. Consistent with this concept, intersectin interacts with the t-SNAREs SNAP23 and SNAP25 [133], and shuttles between active and peri-active zones in an activity-dependent manner [134,135]. Future studies will need to focus on whether intersectin performs a bridge function linking exo- and endocytosis or is rather a multifunctional adaptor participating in these two aspects of SV recycling.

Synapse stability and integrity

Presynaptic AZs must have the capacity to undergo remodeling during periods of synaptic plasticity, but also to remain stable for long-term synaptic maintenance and information storage [136–138]. At present, the mechanisms that control AZ remodeling and stability are poorly understood. Major contributors include cellular systems that control the dynamic exchange and turnover of synaptic proteins [138–140]. These have shown that while most proteins (pre or post) exchange with synapses on the order of tens of minutes to hours, protein turnover is much slower, with most synaptic proteins having a half-life of 2–7 days [139]. This arrangement seems to make sense, as it allows large neurons to simultaneously control the local dynamics of synapses while maintaining sufficient pools of proteins to support activities taking place at great distances from the cell soma. Importantly, while global degradation rates are low, local turnover, regulated by posttranslational modifications such as phosphorylation and ubiquitination that are sensitive to changes in synaptic activity, can be much higher [141,142]. For instance, early proteomic studies demonstrated that synaptic activity could dramatically alter the phosphorylation state of many presynaptic proteins, including Bassoon [143,144], as well as their associations with AZs [145]. Similarly, ubiquitination and the proteasome have been observed to underlie changes in AZ protein composition during homeostatic plasticity [82].

Increasing evidence also implicates degradative pathways, including the ubiquitin–proteasome system (UPS) and the autophagy–lysosomal system, in regulating AZ plasticity and integrity [137]. For example, Liprinα, RIM1, and Munc13 have been shown to undergo degradation via the UPS, through specific ubiquitinating enzymes (anaphase-promoting complex (APC), Scrapper, and FboX45, respectively) that conjugate lysine 48-linked ubiquitin chains to these proteins, targeting them for proteasomal degradation [see 137]. Ubiquitination of RIM1 and Munc13 also modulates neurotransmitter release probability [146–150], suggesting that dynamic ubiquitination of these AZ proteins could be an important mechanism for regulating presynaptic plasticity.

Intriguingly, recent work implicates the two largest CAZ proteins, Piccolo and Bassoon, in regulating the local activity of the UPS and endo-lysosomal degradative pathways [151,152]. Specifically, Piccolo/Bassoon loss-of-function triggers not only the loss of SV pools and synaptic junctions, but also the accumulation of ubiquitinated proteins, pleiomorphic vesicles, and multi-vesicular bodies within degenerating boutons. Importantly, these phenotypes require the activity of both the UPS and endo-lysosomal systems, as well as the E3 ubiquitin ligase Siah1, whose activity is inhibited by its association with the ZnF domains of Piccolo and Bassoon [151]. At present, the function of the AZ-associated degradative system remains rather speculative. One attractive idea is that it performs a surveillance role to facilitate the removal of misfolded/nonfunctional or aging proteins. Alternatively, it could contribute to SV recycling during synaptic transmission. Finally, this system could regulate the growth or elimination of specific synapses during development or periods of synaptic strengthening or weakening. Intriguingly, the phenotypes seen in boutons lacking Piccolo and Bassoon are reminiscent of those seen in animal models of Alzheimer's and Parkinson's diseases [153,154], raising the question of whether local dysregulation of AZ-mediated degradative pathways contributes to the etiology and/or progression of neurodegenerative disease.

Summary

Active zones are highly specialized microdomains designed to regulate neurotransmitter release on a millisecond time scale. The development of super-resolution imaging and cryo-EM technologies is providing unprecedented insights into the nanoscale organization of AZs and their relationship to endocytic zones. These technologies, coupled with emerging strategies to tag-specific domains of synaptic proteins, should allow investigators to elucidate the structures and functions of individual CAZ molecules and define their precise roles in synaptic transmission. Recent work has also begun to shed light on the role of AZs in maintaining synapse integrity, by locally regulating the activity of surveillance systems that monitor protein and organelle health. This new area of investigation will certainly contribute to our understanding of both neurodevelopmental and neurodegenerative disorders.

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Conflict of interest

The authors declare that they have no conflict of interest.

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