

### **Presynaptic terminal differentiation: transport and assembly** Mei Zhen<sup>1</sup> and Yishi Jin<sup>2</sup>

The formation of chemical synapses involves reciprocal induction and independent assembly of pre- and postsynaptic structures. The major events in presynaptic terminal differentiation are the formation of the active zone and the clustering of synaptic vesicles. A number of proteins that are present in the presynaptic active zone have been identified. Recent studies of various mutants have clarified the *in vivo* functions of some of the main players. Time-lapse imaging studies have captured dynamic and transient events in the transport of synaptic components, and therefore provided insight into the early stages of synaptogenesis.

#### Addresses

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### Abbreviations

Bassoon		
CAZ-associated structural protein		
green fluorescent protein		
kinesin motor protein		
mammalian homolog of unc		
Rab3-interacting molecule		
secretion-5		
soluble N-ethylmaleimide-sensitive component attachment		
protein receptor		
synaptic vesicle		
synapse defective		
synaptotagmin		
uncoordinated		
synaptobrevin		

### Introduction

In mature chemical synapses, the ultrastructure of presynaptic terminals consists of densely populated synaptic vesicles forming orderly clusters around the electrondense structures known as the presynaptic active zones. Active zones are tightly associated with the plasma membrane where they are juxtaposed against the postsynaptic density, and their function is to mediate neurotransmitter release. Recycling of synaptic vesicles takes place at the plasma membrane flanking the active zones.

Biochemical purifications have identified three main protein complexes, and each has a key function at the presynaptic active zone [1-4] (Figure 1). The core SNARE (soluble N-ethylmaleimide-sensitive component attachment protein receptor) complex includes syntaxin, synaptobrevin (VAMP), and synaptosomalassociated protein-25 (SNAP25), and is the basic machinery necessary for vesicle docking and fusion with the plasma membrane. A second protein complex that includes mainly Munc18/UNC-18 (mammalian uncoordinated 18/uncoordinated-18), Munc13/UNC-13, and synaptotagmin (syt) interacts with the SNARE complex and regulates the exocytosis of vesicles. The third complex has somewhat variable contents, usually including Piccolo, Bassoon (Bsn), Rab3-interacting molecules (RIMs)/UNC-10, Liprin/synapse defective-2 (SYD-2), CAZ (presynaptic cytomatrix at the active zone)associated structural protein (CAST/ERC), Velis (vertebrate LIN-7 homolog), and Mints (Munc 18-interacting protein)  $[5^{\circ}, 6, 7]$ . This complex is proposed to form a cytomatrix structure at the active zone that tethers vesicles and organizes the distribution of endocytosis and exocytosis machineries. UNC-18/Munc18 and UNC-13/ Munc13 can also be found associated with RIM/UNC-10, Bassoon, and other matrix complexes [6]. Further interactions among the three basic protein complexes through inter- and intra-molecular binding create a highly organized network that underlies the orderly arrangement of presynaptic terminals (Figure 1). As the functions of the SNARE complex in exocytosis have been reviewed extensively, we review here the current progress on the in vivo function of the other active zone proteins and the transport of presynaptic components.

# The functions of cytomatrix proteins at the active zones

Bassoon and Piccolo are two large proteins that share extensive homology (Figure 1, Table 1; [3]). They are present at the active zones of both excitatory and inhibitory synapses, with overlapping expression in most brain regions. The zinc-finger domains of Bassoon and Piccolo can bind to the prenylated Rab3A-associated protein-1 (PRA1) *in vitro* [8]. The central region of Bassoon and Piccolo that contains multiple coiled-coil domains is important for anchoring both proteins to the plasma membrane of the active zone [5<sup>•</sup>]. Piccolo contains additional motifs that allow it to interact with both



#### Figure 1

Schematic of the proteins that are present in the presynaptic cytomatrix at the active zone. Three major protein complexes (see text) have been identified to structure the cytoskeleton near the active zone and to facilitate docking, priming and fusion of synaptic vesicles (Modified with permission from [57]).

cytoskeletal and signaling molecules that regulate vesicle cycling (Table 1; [8–11]). The large size of Bassoon and Piccolo and their interactions with multiple synaptic proteins support their scaffolding role in the cytomatrix at the presynaptic active zone.

Altrock *et al.* and Dick *et al.*  $[5^{\circ}, 12^{\circ}]$  recently examined the *in vivo* functions of Bassoon using a mouse strain carrying a deletion in the central region of the protein. Although homozygous *Bsn* mutant mice are viable and normal at birth, half of them develop epileptic seizures and die with no obvious brain architecture abnormalities. Different types of synapses display variable defects. The total number and morphology of synapses in the hippocampus and cerebellum are not altered. However, excitatory glutamatergic synapses in the hippocampus are partially inactive because of a failure of vesicle fusion. The epileptic seizures in *Bsn* mutant mice are also suggestive of a decrease in the inhibitory synaptic function, although it is unclear whether or not this is a direct effect of the loss of Bassoon function.

In the retina, Bassoon is required for both the formation and the function of photoreceptor synapses. Photoreceptor synapses have specialized active zones that contain a large-surface electron-dense ribbon band extending from the neurotransmitter release site into the cytoplasm. Bassoon is localized to the base of the ribbons close to the release site [13]. The Bsn mutant retina shows a significant reduction in the total number of photoreceptor synapses, and the remaining synapses display structural defects where the ribbons are unable to anchor into the presynaptic active zone region and hence float freely in the cytoplasm. Ectopic ribbon sites and postsynaptic processes are also detected in retinal neurons, which could be a secondary defect triggered by the loss of normal synaptic connectivity. The reduction in the number of synapses and the presence of aberrant active zone structures are compatible with impaired retina function. In Bsn mutant mice, the retina still responds to short light flashes as indicated by an electroretinographic recording (ERG). However, the amplitude and oscillation of certain ERG waves are reduced, which

Table 1			
The structure and <i>in vitro</i> biochemical interactions of the scaffolding active zone proteins.			
Protein	Motifs	Partners	Structure
Bassoon (mammal)	C2H2 zinc-finger	PRA1 (Prenylated Rab3-interacting protein) [1]	CAST
	Coiled-coil (cc)	CAST [7]	Zn         CC1         CC2         CC3         Q           V         PRA1
Piccolo (mammal) CAST/ERC (mammal)	Q domain	Abp1 (actin/dynamin- binding protein) [8]	Abp1 Profilin CAST cAMP-GEFII
	C2H2 zinc-finger	PRA1 [1,11]	
	Coiled-coil (cc)	CAST [7]	
	Other region	GIT (Arf GTPase activating protein) [10]	PRA1 GIT RIM
	Proline rich region	Profilin (actin monomer-binding protein) [1]	
	PDZ domain	cAMP-GEFII (guanine nucleotide	
	C2 domain	exchange factor) [9] RIM [9]	Bassoon/Piccolo
	Coiled-coil (cc)	Liprin [16], Bassoon/Piccolo [7]	
	PDZ-binding site	RIM [6]	Liprin RIM
Liprin-α	Coiled-coil (cc)	RIM [15],	
(mammal)		CAST [16], GIT [58]	KIĘ1A LAR
		KIF1A (kinesin motor	
SVD_2	SAM (storilo	LAR (receptor tyrosine	CC(1-5) SAM(1-3)
(C. elecans)	alpha motif)	nhosnhatase) [19]	RIM CAST GIT
(Drosophila)	domains	phosphatase, [19]	
RIM (mammal)	Zinc-fingers	GTP-Rab3 [15].	
· · · · · (· · <b>·</b> · · · · · · · · · · · · · · · ·	5	Munc-13 [15]	
	PDZ domain	CAST [6]	Rab3A CAST Piccolo Synaptotagmin
UNC-10	C2 domain	Piccolo [9], Synaptotagmin	
(C. elegans)		[15], Liprin [15]	
	Other regions	cAMP-GEFII [60], RIM-BP (Rim-binding protein) [15]	Munc13-1 cAMP-GEFII RIM-BP Liprin

Listed are reported binding partners for the major presynaptic active zone proteins. The structural motifs for each protein are illustrate in the diagrams, and the arrows point to the proteins that bind the specific domain.

suggests that the synaptic transmission from the ribbon synapse is perturbed.

The alteration of synaptic morphology and function in the *Bsn* mutant demonstrates an *in vivo* role for Bassoon in facilitating vesicle exocytosis. The lack of morphological and functional defects in the majority of central nervous system (CNS) synapses could be because the deletion in the central region does not completely abolish the function of Bassoon, or because the loss of Bassoon function is compensated for by Piccolo. Indeed, the expression of Piccolo increases 1.5 times in the *Bsn* mutant [5<sup>•</sup>]. Piccolo mutants have not yet been reported. The differential effect of the loss of Bassoon function is consistent with the heterogeneity of synapses. In photoreceptor ribbon synapses, Bassoon apparently plays a prominent part in synapse formation. The heterogeneity might also exist in subpopulations of the same types of synapses, as only a

fraction of glutamatergic synapses are inactive in the CNS. It is conceivable that synapses in the cerebellum and hippocampus of *Bsn* mutants develop more flexibility to compensate for the loss of individual proteins and individual synapses in the region.

Bassoon and Piccolo associate with another set of active zone proteins that are known as CAST/ERC, Liprin- $\alpha$ / SYD-2, RIM/UNC-10, Munc13/UNC-13, and Munc18/ UNC-18 (Figure 1, Table 1; [7,14<sup>••</sup>]). RIM/UNC-10 and Munc13/UNC-13 regulate exocytosis through their interactions with the core SNARE complex. CAST/ERC, RIM/UNC-10 and Liprin- $\alpha$ /SYD-2 can bind to each other directly *in vitro* [6,15–17]. Co-expression of CAST and Liprin- $\alpha$  in cultured hippocampal neurons enhances the restriction of Liprin- $\alpha$  at the synaptic sites. The loss of function of the liprin proteins in *Caenorhabditis elegans* and *Drosophila* leads to altered active zone morphology, a failure of synapse formation, and reduced synaptic transmission [18,19]. How the CAST/Liprin/RIM complex associates with Bassoon and Piccolo is not well understood. It is also not known if these active zone cytomatrix proteins depend on each other for localization *in vivo*. The physical interactions among these active zone proteins might help to anchor the exocytosis event at the active zone cytomatrix regions through CAST and Liprin. Among the identified active zone components, only Bassoon and Piccolo have no obvious homolog in invertebrates. Therefore, the CAST/Liprin/RIM complex can at least function independently of Bassoon and Piccolo.

# UNC-18/Munc18: regulators of vesicle docking and/or priming?

Synaptic vesicles go through the processes of docking (contact with the plasma membrane at active zones) and priming (become competent for vesicle fusion) before releasing neurotransmitters (Figure 1; [2]). The formation of the core SNARE complex alone is sufficient for the fusion and release of neurotransmitter in vitro, but not in vivo. RIM/UNC-10, Munc13/UNC-13, and Munc18/ UNC-18 are proposed to modulate the formation of the SNARE complex *in vivo* [20]. Whereas UNC-13/Munc13 and UNC-10/RIM modulate the priming of synaptic vesicles by promoting SNARE complex formation in C. elegans, Drosophila, and mouse [15,21-25], the role of Munc18/UNC-18 is more controversial [26]. Weimer et al. [27\*\*] recently demonstrated that UNC-18 plays a part in mediating the docking of synaptic vesicles in C. elegans, where unc-18 is a single gene. In unc-18 mutants, a reduced fraction of synaptic vesicles contact the plasma membrane near the active zone. The reduction in synaptic activity as assessed by physiological recording is more severe than the decrease in the fraction of docked vesicles, which suggests that only some of the docked vesicles are fusion-competent. Syntaxin undergoes conformational change from the 'closed' to 'open' form to allow the formation of the SNARE complex [26]. UNC-18/ Munc18 binds specifically to the closed form of syntaxin [26]. There has been speculation that UNC-18/Munc-18 promotes exocytosis through either the trafficking or the activation of syntaxin. Surprisingly, the role of UNC-18 in C. elegans does not involve syntaxin directly [27<sup>••</sup>]. Although the syntaxin level is reduced by 50% in unc-18 mutants (a phenotype consistent with that of the *Munc18-1* knockout mouse [28]), syntaxin localizes normally in unc-18 mutants. Increasing the level of either a wild type or a constitutively open form of syntaxin in *unc-18* mutants cannot ameliorate the defects in synaptic activity. The increase in the syntaxin levels in unc-18 mutants is also unlikely to restore the docking of vesicles at the active zone. Normal vesicle docking has been observed in studies with Drosophila syntaxin null mutants [23]. The role of UNC-18 in *C. elegans* synapses resembles the function of Munc18-1 in chromaffin cells, where it is required for docking, but not the release of, dense core vesicles [29]. UNC-18/Munc-18 might mediate docking through its physical interaction with other synaptic proteins, such as Doc and Mint [20]. Whether UNC-18/ Munc-18 homologs in other organisms also function independently of or indirectly through syntaxin remains to be examined. Furthermore, the analysis of *C. elegans unc-18* mutants does not rule out the roles of proteins in the family of UNC-18 in priming. For example, in CA1 hippocampal synapses Munc18-1 is required for vesicle fusion, but not docking [28].

# Quantal transport and assembly of the presynaptic active zone

A fundamental issue relating to synapse formation is how the synaptic components get to the synaptic sites. In an early study in this area [30], experimenters using VAMPgreen fluorescent protein (GFP) on cultured hippocampal neurons observed a type of vesicular packet that appeared to contain many cytoplasmic and membrane-associated protein precursors for synaptic vesicles and active zones [30]. Such packets are actively transported in the axons, and are stabilized specifically at sites of synaptic contact. This study has led to the hypothesis that presynaptic components are united early in their biogenesis and sorting pathways, and that membrane remodeling upon synaptic contact subsequently produces the uniform vesicles typical of mature synapses. Recent studies using slightly different methods have revealed additional transporting packets. In particular, the active zone components are transported separately from synaptic vesicles, and are detected earlier than synaptic vesicles at nascent synapses.

Bassoon and Piccolo are among the earliest protein components detected at nascent synapses [31]. Shapira *et al.* [14<sup>••</sup>] recently showed that many components of the protein complexes forming the matrix (Bassoon, Piccolo, CAST) and functional units (RIM/UNC-10, Munc13/ UNC-13 and Munc18/UNC-18) at the active zones could be detected on the same dense core vesicles that are transported along the microtubules to the termini. By fluorescent staining with the antibodies against Piccolo, Bassoon, and RIM, it can be seen that the content, or the level of individual proteins, in a packet appears to vary. Quantification of the protein levels suggests that 2–3 of such active zone packets can deposit sufficient materials to build an active zone at the nascent synaptic termini.

The active zone packets, visualized using Bsn-GFP, appear to be highly motile in young neuronal cells, which coincides with the developmental stage of synaptogenesis [14<sup>••</sup>]. Initially, the Bsn-GFP packets are present in variable sizes, and are not restricted to nascent active zones. During development, the bigger packets with decreased motility are likely to develop into functional synaptic sites. This suggests that prior to synapse formation, the building materials for presynaptic active zones



Transport of presynaptic components. Motor proteins transport different materials or cargoes within the neuron. Some cargoes contain components that are specifically destined for the active zone (black circles and ovals), and some contain synaptic vesicle proteins (gray and green circles). Vesicles from both anterograde transport and local recycling (green and dark blue circles, and endosomes) establish and maintain SV clustering at the synapses. Exocyst (orange and red circles) is required for fusion with plasma membrane of at least one type of anterograde transported vesicle containing SV proteins; and such fusion is necessary for the vesicles to be retained at synapses.

have been pre-assembled and that they can be deposited upon contact with postsynaptic partners to assemble a functional synapse in a timely fashion.

### Synaptic vesicle dynamics: early stage

It is fair to say that the life of a synapse lies in its capacity to accommodate synaptic vesicles and to arrange them in an orderly manner. How are synaptic vesicles generated? Decades of biochemical and cell biological studies have revealed two distinct sources that supply synaptic vesicles (Figure 2; [32]). One is the Golgi apparatus, where vesicles are synthesized de novo and transported by anterograde motor proteins to the synaptic terminals. Such vesicles are usually called synaptic vesicle precursors, because they contain proteins that are often not present in the mature synaptic vesicles. The second source is the local plasma membrane, where synapses are derived through endocytosis of the membrane. Studies in mouse, Drosophila, and C. elegans have provided in vivo confirmation of the two pathways. For example, the loss-of-function mutations in kinesin motor protein (KIF1A) and UNC-104 kinesin motor proteins cause retention of synaptic vesicles and precursors in neuronal cell bodies [33,34]. Mutants that are defective in the genes that function in endocytosis, such as dynamin, synaptojanin, and recently endophilin [35°,36°], exhibit drastic reduction and abnormal arrangement of synaptic vesicles at synapses [37-39]. It remains largely unknown, however, how the synaptic vesicle precursors mature and where the very first source of vesicles that enables the endocytosis pathway comes from. Recent progress in combining time-lapse microscopic imaging in normal and mutant neurons has opened up a valuable venue to begin to address these questions.

How early do functional vesicles appear? An excellent imaging study using VAMP-GFP and FM4-64 (N-[3triethylammoniumpropyl]-4-[6-(4-[diethylamino] phenyl) hexatrienyl] pyridinium dibromide) [40<sup>•</sup>] has observed active vesicular traffic at the growth cone, a stage preceding any synaptic contacts. In the cultured rat visual cortical neurons, VAMP-GFP containing vesicles are present in motile growth cone filopodia. Such vesicles contain a number of synaptic proteins that are found in mature synaptic vesicles. They are able to fuse with the plasma membrane and recycle, raising the possibility that the rare fusion events of such vesicles to plasma membrane might be sufficient to trigger the endocytosis that is necessary for replenishing the synaptic vesicle pool at mature synapses. Furthermore, these vesicles move bidirectionally at a speed that is slower than that of KIF1A, which suggests that the synaptic vesicle (SV) transport might use multiple motors or different motors at different stages (Figure 1).

Further insight into synaptic vesicle biogenesis has come from an elegant genetic analysis on the exocyst complex in the *Drosophila* nervous system. The exocyst is a multisubunit protein complex that is necessary for direct vesicle fusion to specific membrane sites [41,42]. Components of the exocyst complex are present in growth cones [43–45]. Murthy *et al.* [46<sup>••</sup>] generated null mutant flies that lack the exocyst complex because of a deletion in the Sec5 (secretion 5) gene. In these flies, neurons arrest at the neurite outgrowth stage, and neuromuscular junctions fail to grow once maternal Sec5 has run down, which suggests an essential role for exocyst in the addition of new material to the membrane. Using a genetic method that allows them to determine the efficacy of addition of newly synthesized proteins in sec5 null neurons, the authors show that vesicles containing newly synthesized Syt-GFP are present along the length of the axon, but are rarely seen in the synaptic boutons. Yet, as revealed by immunocytochemical and electrophysiological assays, synaptic vesicles are concentrated at the synapses and vesicle exocytosis is normal in Sec5 mutant synaptic boutons. Thus, this study suggests two distinct vesicle fusion mechanisms (Figure 2). Abolishing exocyst function causes a failure of the newly synthesized Syt-GFP labeled vesicles to be retained in the synapses because they cannot fuse with the membrane. It remains to be analyzed how the morphology and the chemical content of the Sec5-dependent Syt-GFP vesicles differ from those of the Sec5-independent synaptic vesicles at the synaptic terminal.

# A new role for $\beta$ -catenin: localizing synaptic vesicles

At mature synapses, synaptic vesicles are divided into three functional pools by their mobility and their electrophysiological capacity: the readily releasable pool (RRP), the resting pool, and the reserve pool [47]. Morphologically, the RRP corresponds to the vesicles docked at the membrane, and the resting and reserve pools to the undocked vesicles. Actin is the major cytoskeletal element involved in tethering the undocked vesicles [48], and the actin binding phosphoprotein synapsin has an essential role in maintaining the reserve pool [49-51]. Using conditional mouse mutants, a recent study has revealed a surprising role of  $\beta$ -catenin in synaptic vesicle localization [52<sup>••</sup>].  $\beta$ -catenin is involved in two classical signaling pathways: the cadherin mediated cell adhesion at membrane junctions in which it interacts with  $\alpha$ -catenin and bridges cadherins to actin, and the Wnt signaling pathway in which it acts along with T cell factor/lymphoid enhancer binding factor (TCF/LEF) to control gene transcription. The involvement of β-catenin at the postsynaptic development and, in particular, in dendritic spine dynamics has been intensely examined in hippocampal neurons, where it functions mainly through its regulation of cadherin-mediated cell adhesion [53]. By ablating the function of β-catenin selectively in hippocampal pyramidal neurons, Bamji et al. [52\*\*] found a 40% reduction in the number of undocked pools of synaptic vesicles at the synapse, accompanied with an increase in the total number of synapses. Time-lapse imaging in the cultured neurons lacking  $\beta$ -catenin reveals that synaptic vesicle proteins are diffusely localized, but that the localization and the number of other presynaptic active zone components including Bassoon and N-cadherin are not affected. These observations suggest that the increase in synapse number is likely to be a compensatory effect to counteract the reduction in synaptic vesicle number. Furthermore, the fluorescent puncta from synaptophysin-GFP exhibit rapid and random movements that are different from the previously reported transport packets.

These results support the conclusion that  $\beta$ -catenin has a specific role in localizing synaptic vesicles during presynaptic assembly. Interestingly, this function of  $\beta$ -catenin does not depend on the domains of  $\beta$ -catenin that are known to interact with  $\alpha$ -catenin or TCF/LEF; rather it requires its PDZ (PSD-95/Discs-large/ZO1) binding domain [52<sup>••</sup>]. The known PDZ domain-binding partners of  $\beta$ -catenin include Veli and SCAM/MAGI-1 (synaptic scaffolding molecule/membrane-associated guanylate kinase with inverted domain organization) [54,55]. Disrupting the Veli PDZ domain has only slight effects on SV localization, which suggests that  $\beta$ -catenin has other PDZ domain binding partners [52<sup>••</sup>].

### Conclusions

With the increasing effort put into generating knockout mutant mice, the in vivo functions of biochemically identified molecules at synapses are being gradually defined and clarified. These studies, together with the information from model invertebrate organisms, have helped us to learn the themes and variations that underlie synapse formation. Combining live imaging analysis with genetic mutant studies provides an invaluable and more accurate assessment of the dynamic cellular process in both wild type and mutant cells. Cells constantly produce synaptic components, which are constantly transported along the axons and are ready to assemble synapses at targeted sites. A plethora of cell surface molecules in target recognition have been identified in recent years [56]. An exciting frontier is how the target recognition event leads to local trapping of the mobile transport materials and how the synaptic precursors mature during the synaptic assembly.

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In this study, the authors confirmed that the active zone transport vesicles contain most of the active zone components by western blotting or immunogold antibody staining. By quantifying the protein level of Bassoon, Piccolo and RIM at the active zone, they concluded that most active zones result from the fusion of 2–3 active zone transport packets. Using the Bassoon-GFP marker, they found that in the early stage of synapse development the active zone transport packets are not restricted to synaptic sites and are highly motile in early stages of synaptogenesis, but are stabilized at the synaptic sites when synapses form.

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In this study, the authors examined the synaptic defects of C. elegans unc-18 mutants by ultrastructural and electrophysiological analysis. They demonstrated that there is a vesicle docking defect in unc-18 mutants. They tested if UNC-18 functions through syntaxin by overexpressing wild type and 'open' form syntaxin in unc-18 mutants. Neither overexpression nor open form syntaxin can rescue the synaptic defects, which suggests that UNC-18 might function through other proteins, or indirectly through svntaxin.

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Please see annotation to Fabian-Fine et al. [36•].

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This study and that of Schuske *et al.* [35<sup>•</sup>] demonstrate an *in vivo* requirement of endophilin in SV endocytosis, and provide further clarification of the mechanisms of endophilin function.

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