

# Cellular and molecular mechanisms underlying presynapse formation

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Synapse formation is a highly regulated process that requires the coordination of many cell biological events. Decades of research have identified a long list of molecular components involved in assembling a functioning synapse. Yet how the various steps, from transporting synaptic components to adhering synaptic partners and assembling the synaptic structure, are regulated and precisely executed during development and maintenance is still unclear. With the improvement of imaging and molecular tools, recent work in vertebrate and invertebrate systems has provided important insight into various aspects of presynaptic development, maintenance, and trans-synaptic signals, thereby increasing our understanding of how extrinsic organizers and intracellular mechanisms contribute to presynapse formation.

Chemical synapses are highly specialized, asymmetric intercellular junction structures that are the basic units of neuronal communication. Proper development of synapses determines appropriate connectivity for the assembly of functional neuronal circuits. Synaptic circuits arise during development through a series of intricate steps (Waites et al., 2005; McAllister, 2007; Jin and Garner, 2008). First, spatiotemporal cues guide axons through complex cellular environments to contact their appropriate postsynaptic targets. At their destination, synapse formation is specified and initiated through adhesive interactions between synaptic partner cells or by local diffusible signaling molecules. Stabilization of intercellular contacts and assembly into functional synapses involves cytoskeletal rearrangements, aggregation, and insertion of pre- and postsynaptic components at nascent synaptic sites. Maturation and modulation of these newly

formed synapses can then occur by altering the organization or composition of synaptic proteins and post-translational modifications to achieve its required physiological responsiveness (Budnik, 1996; Lee and Sheng, 2000). Conversely, retraction of contacts and elimination of inappropriate synaptic proteins help to refine the neuronal circuitry (Goda and Davis, 2003; Sanes and Yamagata, 2009).

Over the last decade, new insights have furthered our understanding of synapse development through the identification of new molecular players and by advanced imaging technology that has allowed for high-resolution inspection of the dynamics and relative positions of synaptic proteins. This review will highlight recent results on the development of presynaptic specializations, and the roles of trans-synaptic organizers, intracellular synaptic proteins, and the cytoskeleton during the formation and maintenance of synapses.

## Axonal transport of synaptic vesicle and active zone proteins

After cell fate determination and morphogenesis, neurons continue to differentiate by entering the phase of synapse formation. Most synaptic material required for this process is synthesized in the cell body of neurons and transported to synapses by microtubule (MT)-based molecular motors (Fig. 1). MTs are intrinsically polarized filaments with a plus and a minus end (Fig. 1 B). MT-based molecular motors use this polarity to transport cargoes to specific cellular locations. Examination of MTs by electron microscopy in dissociated cultured neurons showed that the organizations of MTs is different in axon and dendrite (Baas et al., 1988, 2006). In axons, all microtubules have their minus ends oriented toward the cell body and their plus ends extend distally. On the contrary, the MT polarity in dendrites is mixed. Recent studies tracking the movement of end-binding MT-capping proteins confirmed these results in vivo. Specifically, axonal MTs are uniformly organized with their plus ends pointing distally in all organisms. Dendrites of vertebrate neurons show more plus end–out MTs in vivo, whereas

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Abbreviations used in this paper: Cbln, cerebellin; MAP, MT-associated protein; MT, microtubule; NMJ, neuromuscular junction; PF, parallel fiber; TSP, thrombospondin.

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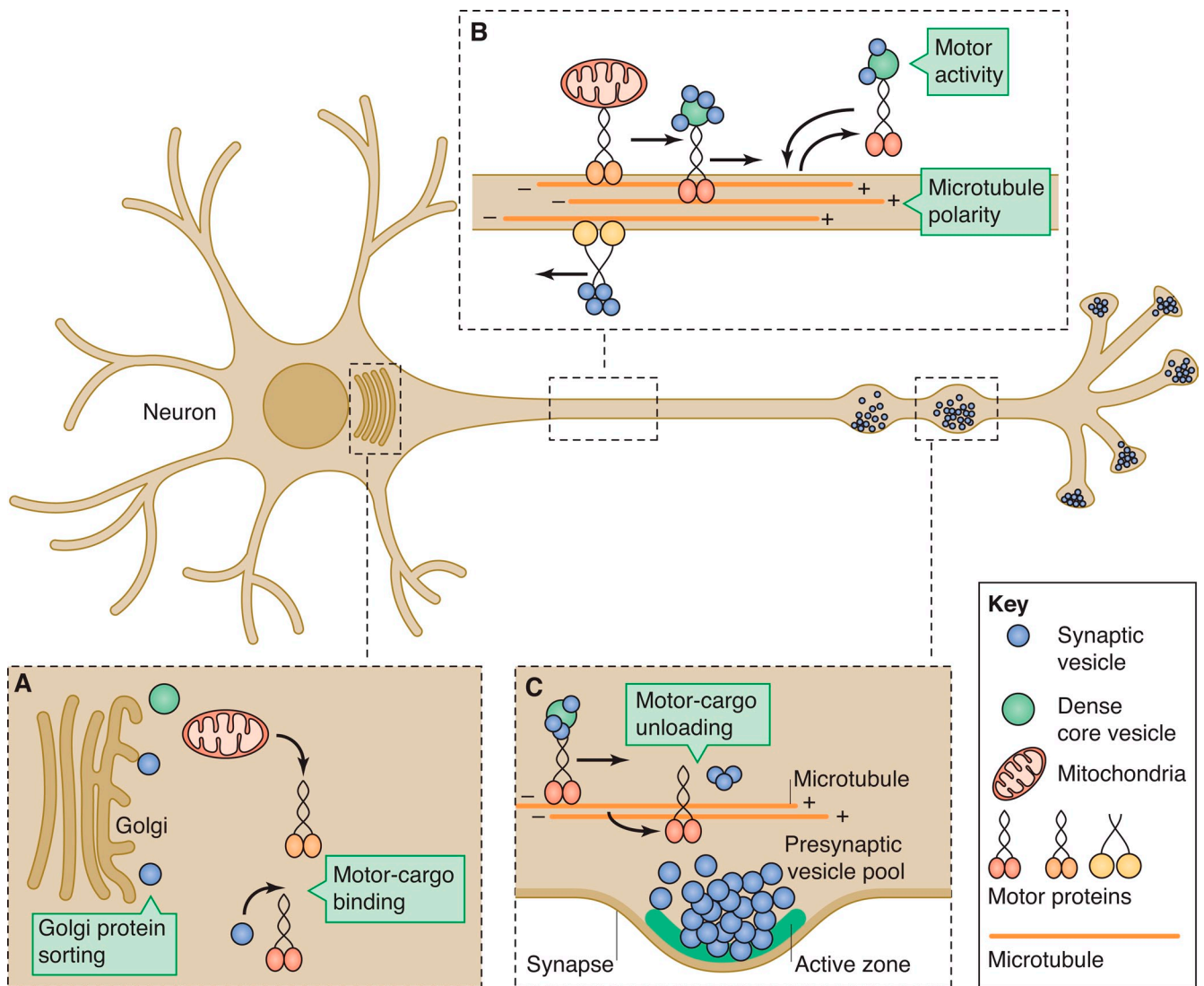


Figure 1. **Regulatory steps during polarized motor-based transport of synaptic material.** (A) At the Golgi apparatus, synaptic proteins have to be sorted into appropriate vesicles. These vesicles and other cargo such as mitochondria get loaded onto specific motor proteins. (B) Establishment of proper microtubule polarity along the axon determines anterograde and retrograde trafficking by plus end- and minus end-directed motor proteins such as kinesins and dynein. (C) At the appropriate destination, motor-cargo unloading occurs in a regulated fashion to achieve the appropriate distribution of synaptic boutons. At synapses, synaptic vesicle precursors give rise to mature synaptic vesicles. Proteins required for the SV cycle and trans-synaptic adhesion coalesce into the active zone (AZ) underneath the plasma membrane juxtaposed against the postsynaptic membrane.

flies and worms have more minus end-out MTs in dendrites (Stepanova et al., 2003; Rolls et al., 2007; Stone et al., 2008).

Does the difference in microtubule organization and polarity help to segregate synaptic cargoes between axons and dendrites? Recent studies have started to identify some molecules that create these differences in MT polarity in different neuronal subcellular compartments and show how disruption of their function affects synapse formation. For example, a recent paper showed that kinesin-1 is required to establish the predominantly minus end-out organization in the dendrites of *Caenorhabditis elegans* motor neurons (Yan et al., 2013). In kinesin-1/*unc-116* mutants, dendrites adopt the axon-like MT polarity causing presynaptic cargoes to mislocalize into dendrites (Seeger and Rice, 2010; Yan et al., 2013). Similarly, loss of the MT-binding CRMP protein UNC-33 or the actin-spectrin adaptor protein ankyrin/UNC-44 in worms also results in MT

polarity defects, which also results in ectopic localization of synaptic vesicles and active zone proteins into dendrites (Maniar et al., 2012). These results support the idea that MT polarity ensures the faithful targeting of presynaptic components to the axon. However, another way motors can distinguish between axons and dendrites is through MT-associated proteins (MAPs). In a recent study, Banker and colleagues showed that plus end-orienting kinesins can differentiate axon and dendrite, likely due to specific MT-binding proteins in these compartments (Huang and Banker, 2012).

The direct regulation of motor activity by MTs or synaptic vesicle-associated proteins is likely to contribute to the trafficking of synaptic cargoes. Doublecortin, a MAP, binds to kinesin-3/KIF1A to affect the trafficking of the synaptic vesicle protein, synaptobrevin, in hippocampal neurons by altering the affinity of ADP-bound KIF1A to MTs (Liu et al., 2012). The Rab3 guanine

nucleotide exchange factor, DENN/MADD, functions as an adaptor between kinesin-3 and GTP-Rab3-containing synaptic vesicles to promote the trafficking of synaptic vesicles in the axon (Niwa et al., 2008).

Precise regulation of motor-based transport ensures that synaptic cargoes are delivered to and maintained at synapses. Several recent studies have provided evidence that two postmitotic cyclin-dependent kinases are important regulators of anterograde and retrograde trafficking of presynaptic cargoes. The kinase CDK-5 is required in many aspects of nervous system function. In the context of presynaptic development and function, CDK-5 has been shown to regulate the transport of synaptic vesicles and dense core vesicles, which contain neuropeptides, by inhibiting a dynein-mediated pathway that mobilizes presynaptic components to the somatodendritic compartments in *C. elegans* neurons (Ou et al., 2010; Goodwin et al., 2012). A paralogue of CDK-5, the PCT-1 kinase acts in a partially redundant pathway to prevent the mislocalization of presynaptic material to dendrites. In animals lacking both kinases or their activators, synaptic cargoes completely mislocalize to the dendrites, leaving an “empty” axon (Ou et al., 2010). Vertebrate CDK-5 also plays profound roles in the regulation of synaptic vesicle pools by modifying Ca<sup>2+</sup> channels. Genetic ablation or pharmacological inhibition of CDK-5 increases the pool of synaptic vesicles that are docked at the active zone, termed the readily releasable pool, and potentiates synaptic function (Kim and Ryan, 2010, 2013). These results suggest that CDK-5 and its paralogue control local and global vesicle pools. Regulation of the exchange between these pools can affect membrane trafficking at presynaptic terminals as well as the overall polarity of neurons.

To form synapses at defined locations, cargoes not only need to know how to “get on” the transport system but also need to know where to precisely “get off” at their destination (Fig. 1 C). Loss of a conserved small G-protein of the Arf-like family, ARL-8, in *C. elegans*, resulted in premature exit of synaptic cargoes during transport and showed ectopic aggregations of synaptic vesicles in the proximal axon. This causes a reduction in the number but an increase in the size of synapses (Klassen et al., 2010). ARL-8 localizes to both stable and trafficking synaptic vesicles and promotes trafficking by increasing kinesin-3 activity and suppressing aggregation-induced stoppage of synaptic cargoes along the axon (Wu et al., 2013). Hence, the balance between motor activity and aggregation propensity of trafficking cargoes may determine the number, size, and location of presynaptic terminals. Interestingly, the small GTPase Rab3, which normally associates with synaptic vesicles, has recently been shown to affect the distribution of active zone proteins at fly neuromuscular junction (NMJ) synapses, further suggesting that the trafficking of synaptic vesicles and formation of active zones are linked (Graf et al., 2009).

Besides synaptic material, another major organelle cargo that is often present at the presynaptic terminal is mitochondria. The Milton–Miro complex functions as an adaptor between kinesin-1 and mitochondria to support axonal transport of mitochondria. Interestingly, the coupling of the Milton–Miro complex to kinesin is regulated by Ca<sup>2+</sup> (Macaskill et al., 2009; Wang and

Schwarz, 2009), providing a mechanism for neuronal activity controlling transport of mitochondria along the axon.

Previous studies have suggested that components of the presynaptic active zone are transported in a preassembled form by Piccolo-Bassoon transport vesicles (PTVs) that may contain multiple components required to build a synapse (Zhai et al., 2001; Shapira et al., 2003). Recent studies found that Golgi-derived PTVs contain many active zone proteins including Piccolo, Bassoon, RIM1 $\alpha$ , and ELKS2/CAST, but lack another active zone component, Munc-13, which may exit the Golgi on separate vesicles (Maas et al., 2012). Packing of various active zone components that have the propensity to self-assemble into separate vesicles may contribute a way to control synaptogenesis. This is interesting in light of the finding that Munc-13 can function as a protein scaffold for Bassoon and ELKS2 (Wang et al., 2009). The link between trafficking of synaptic vesicle and active zone components is not well understood. In vivo time-lapse imaging of synaptic vesicle and active zone trafficking showed that these components, possibly in the form of dense core vesicles, could be trafficked together in *C. elegans* neurons, suggestive of prepackaged presynaptic material during transport (Wu et al., 2013). Taken together, axonal transport of synaptic components is a necessary step for synapse formation and maintenance. The regulation of MTs, molecular motors, and synaptic cargoes ensure the targeting of appropriate proteins to synapses.

### Role of the actin cytoskeleton in presynaptic assembly

Although MT-mediated transport is critical for long-range trafficking, actin-based mechanisms often organize local protein complexes in subcellular domains. A large body of work has described the role of the actin cytoskeleton in postsynaptic structure and function (Schubert and Dotti, 2007; Hotulainen and Hoogenraad, 2010). We will focus on more recent work that has highlighted the importance of the actin cytoskeleton in presynaptic formation.

F-actin is required for presynaptic assembly during the early stages of synaptogenesis. Depolymerization of F-actin in young hippocampal neuronal cultures results in a reduction in the size and number of synapses. This effect was not seen with older cultures when synapses are more mature (Zhang and Benson, 2001). This observation correlates with an increase in both pre- and postsynaptic F-actin levels in newly formed synapses compared with mature synapses (Zhang and Benson, 2002).

F-actin has been implicated in many steps of synapse assembly and function (Fig. 2; Cingolani and Goda, 2008). One of the roles that has been proposed for F-actin is to act as a scaffold for other presynaptic proteins (Sankaranarayanan et al., 2003). A recent study identified an F-actin-binding active zone molecule Neurabin/NAB-1 that is recruited by a presynaptic F-actin network (Chia et al., 2012). In addition, knockdown of Rac/Cdc42 GTPase exchange factor  $\beta$ -Pix resulted in a decrease in actin at synapses with a concomitant loss of synaptic vesicle clustering (Sun and Bamji, 2011). These studies demonstrate that F-actin at presynaptic sites can recruit and stabilize presynaptic components.

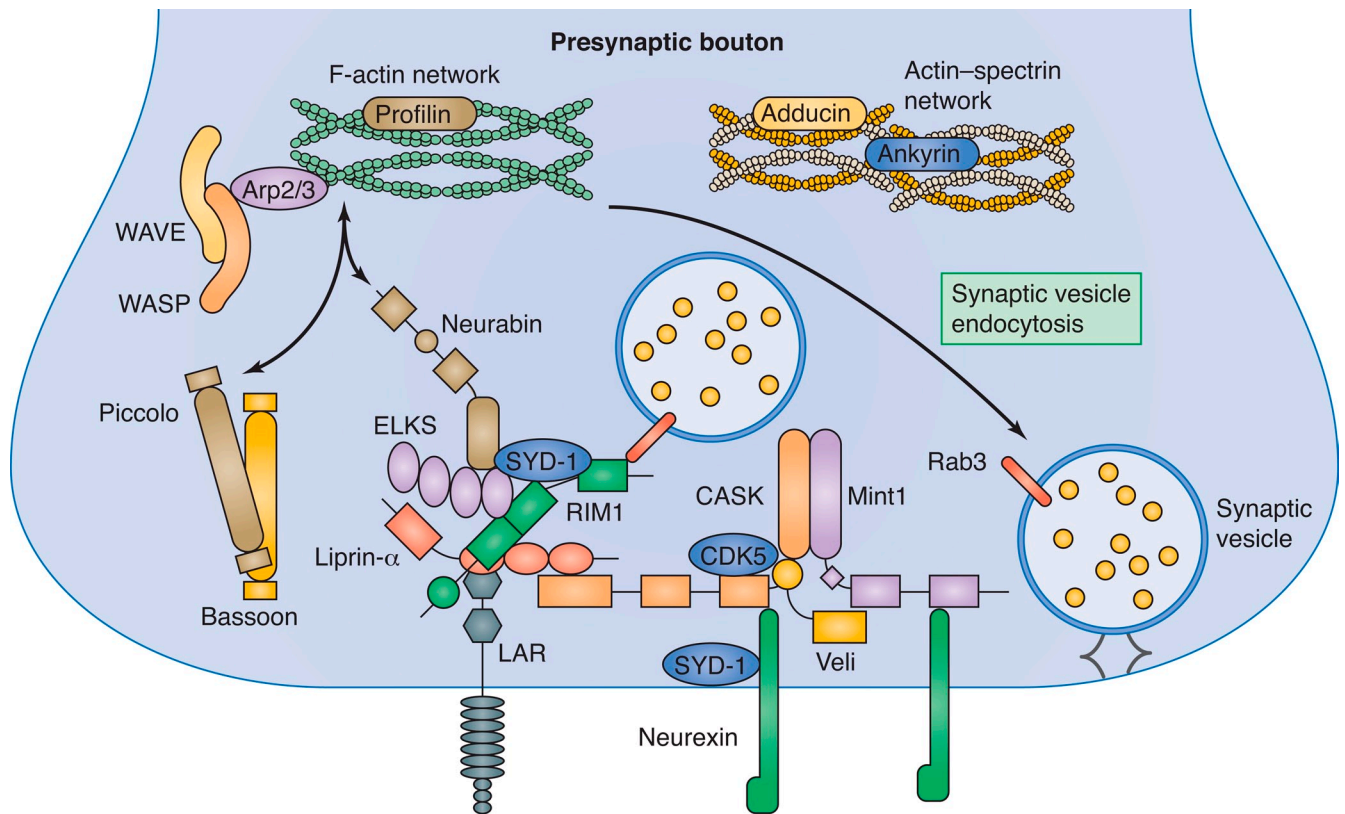


Figure 2. **Assembling the presynaptic active zone.** Scaffolding proteins including Liprin, SYD-1, ELKS, Neurabin, Piccolo, and Bassoon form the dense protein network in the presynaptic cytomatrix that facilitates synaptic vesicle docking and fusion. The presynaptic F-actin networks are required for presynaptic assembly and maintenance.

Studies of *Drosophila* NMJs have found that the presynaptic spectrin–actin cytoskeleton is important for synapse stability. Loss of presynaptic spectrin led to retraction of synapses (Pielage et al., 2005). Intriguingly, loss of postsynaptic spectrin increased the total number of the active zone specializations, termed T-bars, and affected the size and distribution of presynaptic sites. Thus, the spectrin cytoskeleton can impose a trans-synaptic influence on synapse development (Pielage et al., 2006).

Given the importance of F-actin at synapses, it is crucial to understand the signaling pathways that instruct F-actin organization. Multiple studies have shown that signaling from synaptic cell adhesion molecules can lead to cytoskeletal rearrangements at synapses. Adhesion of hippocampal neurons to syndecan-2-coated beads is sufficient to induce F-actin clustering and downstream formation of presynaptic boutons (Lucido et al., 2009). In mice, the adhesion molecule LICAM may bind to spectrin–actin adaptor ankyrin to mediate GABAergic synapse formation (Guan and Maness, 2010). Another adhesion molecule of the immunoglobulin superfamily SYG-1 in *C. elegans* has also been shown to be necessary and sufficient to recruit F-actin to synapses (Chia et al., 2012). In a recent study, secreted bone morphogenetic protein (BMP) can signal in a retrograde fashion to regulate Rac-GEF Trio expression in presynaptic neurons, which is important for controlling synaptic growth (Ball et al., 2010).

Interestingly, presynaptic active zone proteins can also affect F-actin assembly (Fig. 2). Knockdown of Piccolo reduced activity-dependent assembly of F-actin at synapses and enhanced dispersion of Synapsin1a and synaptic vesicles in hippocampal

neurons. Loss of Piccolo also resulted in a loss of Profilin 2, a regulator of actin polymerization (Waites et al., 2011).

Various studies have begun to shed light on the actin regulators required for synaptic F-actin establishment and maintenance. *Diaphanous*, a formin-related gene that associates with barbed ends of F-actin, was found to function downstream of presynaptic receptor *Dlar* at fly NMJs. Spectrin–actin capping protein, Adducin, is enriched at presynaptic sites and is required to prevent synapse retraction and elimination (Bednarek and Caroni, 2011; Pielage et al., 2011). Activators of the Arp2/3 complex, WASP and WAVE, have also been implicated in the regulation of F-actin at synapses (Coyle et al., 2004; Stavoe et al., 2012; Zhao et al., 2013). This diversity of F-actin modulators suggests that there are probably different F-actin structures at different stages of development or even in subcellular domains within the synapse. This is supported by observations that F-actin can localize with synaptic vesicles, at the active zone and in the perisynaptic region (Bloom et al., 2003; Sankaranarayanan et al., 2003; Waites et al., 2011; Chia et al., 2012). Thus, much remains to be done in our understanding how distinct F-actin structures are formed and regulated to mediate various processes during synapse assembly and maintenance.

#### Assembly of the molecular network at presynaptic terminals

Although F-actin might help to initiate the presynaptic assembly process, many other ensuing molecular interactions are required to form the mature presynaptic apparatus (Fig. 2). The

presynaptic active zone is comprised of a framework of scaffolding proteins that function as protein-binding hubs for other presynaptic components. Piccolo and Bassoon are important vertebrate multidomain proteins that traditionally have been widely used as active zone markers. Recent electrophysiology data on Piccolo mutant and Bassoon knockdown neurons showed that these molecules are dispensable for synaptic transmission but affect synaptic vesicle clustering (Mukherjee et al., 2010). Furthermore, Piccolo and Bassoon were found to be required for maintaining synapse integrity by regulating ubiquitination and degradation of presynaptic components (Waites et al., 2013).

Forward genetic approaches in worms and flies have made important contributions to our understanding of the presynaptic cytomatrix. Studies have found that two active zone scaffolding molecules, SYD-1 and Liprin- $\alpha$ /SYD-2, are required for proper synapse formation (Zhen and Jin, 1999; Patel et al., 2006; Astigarraga et al., 2010; Oswald et al., 2010; Stigloher et al., 2011). Interestingly, at fly NMJs, SYD-1 is necessary for clustering presynaptic neuroligin that in turn clusters postsynaptic neuroligin (Oswald et al., 2012). The presynaptic assembly function of SYD-1 and SYD-2 appears to be conserved because mutation analysis of mammalian SYD-1 and knockdown of Liprin- $\alpha$  both caused defects in presynaptic development and function (Spangler et al., 2013; Wentzel et al., 2013). In flies, the active zone T-bar structure is comprised of ERC/CAST family protein *bruchpilot* (*brp*) as the major active zone organizing protein (Fouquet et al., 2009). Brp is not only present at the active zone but also plays important scaffolding roles in localizing Ca<sup>2+</sup> channels. In *C. elegans*, the Brp homologue ELKS-1 is also localized to the active zone; however, the importance of ELKS-1 during development of synapses was only revealed in sensitized genetic backgrounds (Dai et al., 2006; Patel and Shen, 2009), suggesting that there are likely redundant molecular pathways for presynaptic assembly. In the vertebrate system, loss of one of the three ELKS genes, surprisingly, caused an increase in the inhibitory synaptic transmission (Kaeser et al., 2009). Besides Brp, Rab3-interacting molecule (RIM) binding protein (RBP) was found to be important for active zone structural integrity in flies. Using super-resolution microscopy, RBP was found to surround Ca<sup>2+</sup> channels at T-bars and loss of RBP resulted in defective Ca<sup>2+</sup> channel clustering and reduced evoked neurotransmitter release (Liu et al., 2011).

Assembly of the presynaptic active zone is subjected to several layers of regulation. The assembly process is balanced by inhibitory mechanisms that control the number and size of synapses. Loss of the E3 ubiquitin ligase Highwire/RPM-1 results in an increased number of synaptic boutons in flies and multiple active zones in worms (Wan et al., 2000; Zhen et al., 2000). Working together with F-box protein FSN-1, RPM-1 down-regulates the DLK MAP kinase signaling pathway (Liao et al., 2004; Nakata et al., 2005; Yan et al., 2009). Another E3 ubiquitin ligase, the SKP complex, has been shown to eliminate transient synapses during development in worms (Ding et al., 2007). Therefore, ubiquitin-mediated mechanisms play important roles in controlling the presynaptic assembly program.

Other inhibitory mechanisms include SRPK79D, a serine-arginine protein kinase discovered in flies that represses T-bar formation (Johnson et al., 2009). In the mutant, the T-bar component Brp is ectopically accumulated in the axonal shaft. Regulator of synaptogenesis, RSY-1, limits the extent of presynaptic assembly by directly binding to active zone scaffold molecule Liprin- $\alpha$ /SYD-2 and SYD-1 (Patel and Shen, 2009). In addition, Liprin- $\alpha$ /SYD-2 may inhibit its own activity via intramolecular interactions (Taru and Jin, 2011; Chia et al., 2013).

Taken together, the presynaptic assembly process driven by scaffolding molecules is controlled by complex inhibitory mechanisms to achieve the appropriate extent of aggregation in the process of synapse formation.

### **Trans-synaptic signals orchestrate pre- and postsynaptic formation**

Coordinated pre- and postsynaptic development requires the precise apposition of presynaptic components to postsynaptic specializations. It is conceivable that signals from pre and postsynaptic sides functioning across the synaptic cleft coordinate synaptic differentiation reciprocally. Although a vast assortment of factors have been identified as synaptic organizers, the fact that genetic ablation of some synaptic organizers *in vivo* fails to elicit dramatic synaptic defects suggests the incomplete view of the trans-synaptic signaling. Moreover, the underlying mechanisms and the cross talk of these signaling pathways are still unclear. In recent years, an emerging body of literature has begun to shed light on trans-synaptic signaling and the importance of environmental cues in synapse formation.

### **Adhesion proteins instruct synaptic differentiation**

A large body of literature suggests that trans-synaptic interactions between synaptic adhesion molecules function bi-directionally for synapse formation and maturation (Fig. 3). Neuroligin-neuroligin is the first pair to be shown to induce pre- and postsynapse formation (Scheiffele et al., 2000; Graf et al., 2004; Chih et al., 2005; Nam and Chen, 2005; Chubykin et al., 2007). Recent *in vitro* studies have unveiled more components interacting with neuroligin or neuroligin in specific synaptic differentiation events (Fig. 3, B and C). In early developmental stages, a secreted synaptic organizer, thrombospondin 1 (TSP1, see next section) increases the speed of synaptogenesis through neuroligin 1 (Xu et al., 2010). At excitatory synapses, a retrograde signaling controls synaptic vesicle clustering, neurotransmitter release, and presynaptic maturation by cooperation of neuroligin and N-cadherin (Wittenmayer et al., 2009; Stan et al., 2010; Aiga et al., 2011). A leucine-rich repeat transmembrane (LRRTM) protein family was also identified as an organizer of the function of excitatory synapses through interactions with neuroligin (Linhoff et al., 2009). Further studies showed that binding of LRRTMs and neuroligins to neuroligin acts redundantly to maintain excitatory synapses by preventing activity and Ca<sup>2+</sup>-dependent synapse elimination during early development, while performing divergent functions upon synapse maturation (de Wit et al., 2009; Ko et al., 2009, 2011; Soler-Llavina et al., 2011).

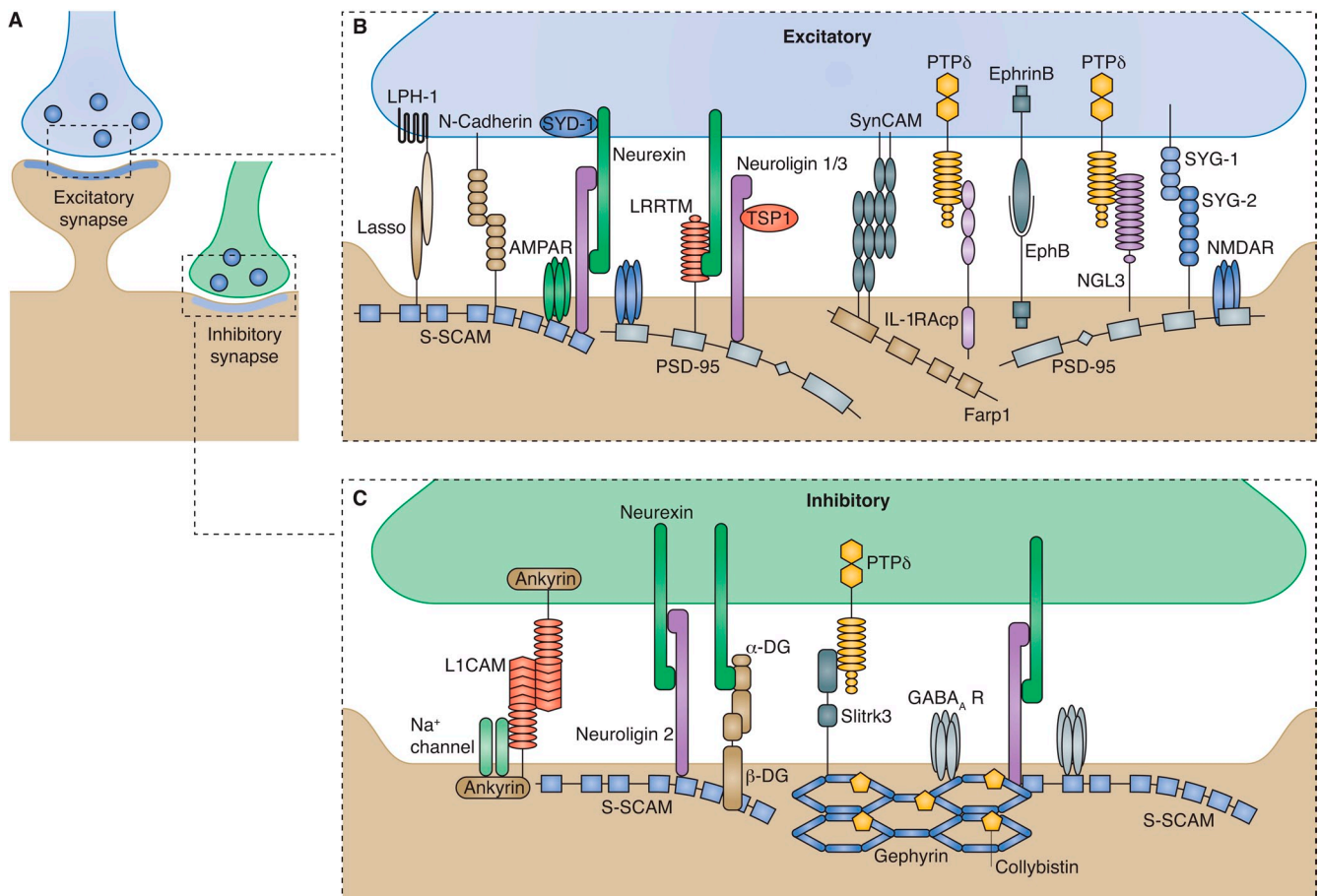


Figure 3. **Adhesive trans-synaptic signaling molecules orchestrate excitatory and inhibitory synaptic assembly.** Multiple pairs of trans-synaptic adhesion molecules organize synaptic differentiation and function on both pre- and postsynaptic sites. Note that different adhesion molecules are used at excitatory and inhibitory synapses. LPH1, latrophilin 1;  $\alpha$ -DG,  $\alpha$ -dystroglycan;  $\beta$ -DG,  $\beta$ -dystroglycan; S-SCAM, synaptic scaffolding molecule; Lasso, LPH1-associated synaptic surface organizer; IL-1RAcP, interleukin-1 receptor accessory protein.

The function of neurexin and neuroligin in mediating synaptic differentiation has also been shown at *Drosophila* NMJs and mammalian CNS. In mammalian, although neither compound knockout of three neurexins nor two individual neuroligin knockout mice display severe defects in the number or morphology of synapses (Missler et al., 2003), the deletion of either neurexin or neuroligin affects the neurotransmitter release and in turn impairs the relevant behavior (Zhang et al., 2005; Blundell et al., 2009, 2010; Etherton et al., 2009; Jedlicka et al., 2011). Neurexin loss of function in fly leads to reduced number and defective morphology of synaptic boutons and active zones from early developmental stages (Li et al., 2007; Chen et al., 2010). In contrast, deletion of either neuroligin 1 or 2 causes NMJ defects and alternations of active zones only in the larval stage, indicating that they function mainly in the expansion of NMJs during development (Banovic et al., 2010; Sun et al., 2011). These abnormalities further impair synaptic transmission at the NMJs (Li et al., 2007; Banovic et al., 2010; Chen et al., 2010; Sun et al., 2011). Moreover, these phenotypes are enhanced when the Teneurin family of adhesion molecules is deleted, suggestive of functional redundancy between adhesion molecules (Mosca et al., 2012). Recently, it has been reported that an active zone protein, SYD-1, is required for the

formation and function of the neurexin–neuroligin complex in flies (Fig. 2; Oswald et al., 2012), providing an example of how trans-synaptic neurexin–neuroligin signaling orchestrates synaptic assembly bi-directionally. Interestingly, at postsynaptic sites, the NMDA receptor activity-triggered  $Ca^{2+}$ -dependent cleavage of neuroligin 1 was found to destabilize presynaptic neurexin, reduce presynaptic release probability, and depress synaptic transmission (Peixoto et al., 2012). This observation raises a possibility that neurexin and neuroligin could fine-tune synaptogenesis both positively and negatively.

Although *Drosophila* neuroligin and neurexin mutants share many phenotypes in synaptic differentiation, there are some unique features for each mutant, suggesting that they play distinct roles. For example, some aspects of synaptic specificity are achieved by different pairs of neurexin–neuroligin interactions. Neuroligin 1 promotes the growth and differentiation of excitatory synapses by binding to PSD-95, whose amount balances the ratio of excitatory-to-inhibitory synaptic specializations (Prange et al., 2004; Banovic et al., 2010). Neuroligin 2, on the contrary, binds to a scaffold protein gephyrin at inhibitory synapses, instructing inhibitory postsynaptic assembly (Fig. 3, B and C; Pouloupoulos et al., 2009). Different isoforms of neurexin also contribute to the differentiation of excitatory

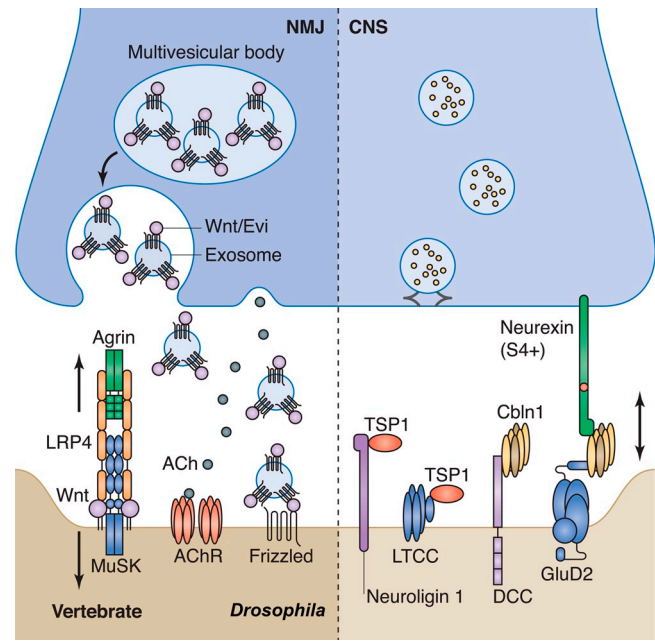
and inhibitory synapses (Fig. 3, B and C; Chih et al., 2006; Graf et al., 2006; Kang et al., 2008).

Other novel trans-synaptic interactions have also been identified to organize synaptic differentiation (Fig. 3, B and C). For example, Netrin-G ligand 3 (NGL-3), localized at postsynaptic region, induces excitatory synaptic differentiation by interacting with the receptor tyrosine phosphatase LAR family proteins, including PTP $\delta$  and PTP $\sigma$  (Woo et al., 2009; Kwon et al., 2010). PTP $\delta$  can also trans-interact with Slitrk3 and IL-1 receptor accessory protein (IL-1RAcP) to promote presynaptic formation (Takahashi et al., 2012; Yoshida et al., 2012). Molecules that function in other neuronal developmental processes have also been shown to regulate synaptic differentiation. Farp1, essential for the dynamics of dendritic filopodia, regulates postsynaptic development and triggers a retrograde signal promoting active zone assembly by binding to SynCAM 1 (Cheadle and Biederer, 2012). Teneurins, instructing synaptic partner selection in fly olfactory system (Hong et al., 2012), act in synaptogenesis through trans-synaptic interaction at NMJs (Mosca et al., 2012). Another splice variant of a postsynaptic Teneurin-2 in rat, Lasso, binding with presynaptic Latrophilin 1 (LPH1), induces presynaptic Ca<sup>2+</sup> signals and regulates synaptic function (Silva et al., 2011). Neural activity is also involved in controlling the growth of the presynapse. Conditioning or BDNF application induces presynaptic bouton development via an ephrin-B-dependent manner (Li et al., 2011), suggesting the role of EphB/ephrin-B signaling in activity-dependent synaptic modification.

### Secreted molecules organize synapse differentiation

In addition to adhesion molecules, some secreted molecules also serve as synaptic organizers (Fig. 4). For example, the motor neuron-derived ligand agrin, which was the first identified secreted organizing molecule for postsynaptic differentiation, activates MuSK, a postsynaptic receptor tyrosine kinase, to regulate NMJ specialization (Glass et al., 1996; Zhou et al., 1999). Recently, a low-density lipoprotein receptor-related protein, LRP4, was identified as the co-receptor of agrin, forming a complex with MuSK and mediating MuSK signaling (Kim et al., 2008; Zhang et al., 2008). Several Wnts appears to act together with agrin to activate the LRP4–MuSK receptor complex to promote postsynaptic differentiation (Jing et al., 2009; Zhang et al., 2012). LRP4 also acts as a direct retrograde signal, functioning independently of MuSK for presynaptic differentiation (Yumoto et al., 2012), demonstrating that LRP4 acts as a bi-directional synaptic organizer (Fig. 4, left).

Wnt is another well-characterized signaling molecule regulating many developmental processes including synaptic differentiation bi-directionally. Wnt regulates synaptic assembly both positively and negatively. For example, Wnt3 collaborates with agrin to promote the clustering of acetyl choline receptor (AChR) at the vertebrate NMJs (Henriquez et al., 2008), while Wnt3a inhibits AChR aggregation through  $\beta$ -catenin signaling (Wang et al., 2008). In the *C. elegans* NMJ, a Wnt molecule, CWN-2, stimulates the delivery and insertion of AChR to the postsynaptic membrane through the activation of a



**Figure 4. Secreted trans-synaptic signaling at NMJs and CNS synapses.** (Left) At *Drosophila* neuromuscular junctions (NMJs), Wnts are secreted from presynaptic terminals in association with Evi in the form of exosomes. In vertebrate NMJs, Wnt binds to the Agrin–LRP4–MuSK complex to regulate synapse formation. (Right) At CNS synapses, glia-derived thrombospondins (TSPs) and presynaptic neuron-derived cerebellin (Cbln) organize synapse differentiation and formation bi-directionally through binding to GluD2 and an isoform of neurexin (S4+) on the postsynaptic and presynaptic membranes, respectively. LTCC, L-type Ca<sup>2+</sup> channel complex; AChR, acetyl choline receptor.

Frizzled–CAM-1 receptor complex (Jensen et al., 2012). Local Wnt gradient can suppress synapse formation in both *C. elegans* and *Drosophila* (Inaki et al., 2007; Klassen and Shen, 2007). Interestingly, in these contexts, Wnts are secreted from nonneuronal or nonsynaptic partner cells, suggesting that environmental factors can shape synaptic connections. Wnt can also be secreted from presynaptic neurons. A recent study demonstrated the trans-synaptic transmission of Wnt by exosome-like vesicles containing the Wnt-binding protein Evi at *Drosophila* NMJs (Fig. 4, left; Korkut et al., 2009; Koles et al., 2012). Presynaptic vesicular release of Evi is required for the secretion of Wnt. Intriguingly, different Wnt ligands regulate synapse formation in distinct cellular contexts. Wnt3a promotes excitatory synaptic assembly through CaMKII, whereas Wnt5a mediates inhibitory synapse formation by stabilizing GABA<sub>A</sub> receptors (Cuitino et al., 2010; Ciani et al., 2011). This functional diversity indicates that different Wnts, receptors, and downstream pathways, as well as cell-specific contexts dictate the action of extracellular cues. Another conserved secreted molecule, netrin/UNC-6, can also pattern synapses by either promoting or inhibiting synapse formation (Colón-Ramos et al., 2007; Poon et al., 2008). Because Wnt and netrin often exist in gradients, these observations suggest that the localization of synapses can be specified by the gradient of extrinsic cues.

In mammalian, several glia-derived cues have been shown to play important roles in regulating synapse formation or elimination. Thrombospondins (TSPs) are trans-synaptic organizers

secreted from immature astrocytes (Christopherson et al., 2005). Both in vitro and in vivo data demonstrate the capacity of TSPs to increase synapse number, promote the localization of synaptic molecules, and refine the pre- and postsynaptic alignment (Christopherson et al., 2005; Eroglu et al., 2009). Recently, two transmembrane molecules were uncovered in mediating TSP-induced synaptogenesis (Fig. 4, right). Neuroligin 1 interacts with TSP1 with its extracellular domain mediating the acceleration of synaptogenesis in hippocampal neurons (Xu et al., 2010).  $\alpha 2\delta$ -1, a subunit of the L-type  $\text{Ca}^{2+}$  channel complex (LTCC), was also identified as the postsynaptic receptor of TSP in excitatory CNS neurons (Eroglu et al., 2009). Interaction between TSP and  $\alpha 2\delta$ -1 triggers the conformational changes and sequentially recruits synaptic scaffolding molecules and initiates synapse formation (Eroglu et al., 2009). Interestingly, TSP-induced synapses, although structurally normal and presynaptically active, are postsynaptically silent due to the lack of AMPA receptors (Christopherson et al., 2005), indicating the existence of other glia-derived signals involved in synapse formation. In fact, in cultured hippocampal neurons, a glia-derived neurotrophic factor GDNF enhances the pre- and postsynaptic adhesion by triggering the trans-homophilic interaction of its receptors GFR $\alpha$ 1 localized at both pre- and postsynaptic sites (Ledda et al., 2007). Several other glia-derived factors have been shown to play critical roles in synaptogenesis. Astrocytes secrete extracellular molecules hevin and SPARC to regulate synapse formation in vitro and in vivo (Kucukdereli et al., 2011). Astrocytes also express a transmembrane adhesion protein, protocadherin- $\gamma$ , serving as a local cue to promote synapse formation (Garrett and Weiner, 2009). TGF- $\beta$  secreted from the NMJ glia acts together with the muscle-derived TGF- $\beta$  to control synaptic growth (Fuentes-Medel et al., 2012). In a similar fashion, secretion of BDNF by vestibular supporting cells is required for synapse formation between hair cells and sensory organs (Gómez-Casati et al., 2010).

Another important synaptic organizer is cerebellin (Cbln), a presynapse-derived complement protein, C1q-like family protein. In *cbln1*-null mice the number of parallel fibers (PF)–Purkinje synapses is dramatically reduced; the postsynaptic densities in the remaining synapses are larger than the apposite active zones (Hirai et al., 2005). Cbln was also found to regulate synaptic plasticity, as *cbln1*-null mice show impaired long-term depression in cerebellum (Hirai et al., 2005). These defects precisely resemble those in mice lacking a putative glutamate receptor, *GluD2* (Kashiwabuchi et al., 1995; Kurihara et al., 1997), suggesting that Cbln1 and GluD2 function in synaptic differentiation through a common pathway. Interestingly, the C-terminal domain and N-terminal domains of GluD2 are indispensable for cerebella LTD and PF–Purkinje synaptic morphology, respectively (Kohda et al., 2007; Uemura et al., 2007; Kakegawa et al., 2008, 2009). Further studies suggested that Cbln1 directly binds to the N-terminal domain of GluD2 and recruits postsynaptic proteins by clustering GluD2 (Matsuda et al., 2010). Neurexin was recently reported as the presynaptic receptor of Cbln in promoting synaptogenesis (Uemura et al., 2010), which reinforces the understanding of Cbln-mediated trans-synaptic signaling: Cbln serves as a bi-directional synapse organizer

by linking presynaptic neurexin and postsynaptic GluD2 (Fig. 4, right).

Besides being required for synapse formation at early stages, genetic ablation of GluD2 in adult cerebellum leads to loss of PF–Purkinje synapses (Takeuchi et al., 2005), indicating that Cbln1–GluD2 signaling is also important for the maintenance of PF–Purkinje synapses. Chronic stimulation of neural activity decreases *Cbln1* expression and diminishes the number of PF–Purkinje synapses (Iijima et al., 2009), suggesting the importance of Cbln1–GluD2 signaling for synaptic plasticity and homeostasis.

Cbln subfamily proteins are widely expressed throughout the brain (Miura et al., 2006), suggesting that their synaptogenic roles may be wide spread in other regions of the brain. Cbln2 and 4 are also secreted proteins, whereas Cbln3 is retained in the cellular endomembrane system (Iijima et al., 2007). Cbln1 and 2, interacting with an isoform of presynaptic neurexin, induce synaptogenesis (Joo et al., 2011; Matsuda and Yuzaki, 2011). Notably, the cortical synapses induced by neurexin–Cbln signaling are preferentially inhibitory (Joo et al., 2011), distinguishing the effects of Cbln from neuroligin. GluD1 was recently found to be the postsynaptic receptor of Cbln1 and 2 in cortical neurons, mediating the differentiation of inhibitory presynapses (Yasumura et al., 2012). On the other side, Cbln4 selectively binds to the netrin receptor DCC in a netrin-displaceable manner (Fig. 4, right), suggesting a potential function of Cbln4 through DCC signaling pathway (Iijima et al., 2007). Intriguingly, C1q, although sharing similar structure with Cbln, serves an opposite role by regulating the synapse elimination: C1q released from retinal ganglion cells refines the retinogeniculate connections by eliminating unneeded synapses (Stevens et al., 2007).

### Concluding remarks

Synapse development is regulated in multiple steps. Research over the last few years have uncovered many regulatory mechanisms on how trafficking of synaptic material is regulated and how scaffold proteins act with cytoskeleton networks and trans-synaptic signaling to instruct the synapse formation. Nevertheless, our understanding of the cellular and molecular mechanisms regulating synapse development is still incomplete. For example, how is the direction, speed, and amount of synaptic material being transported specified? How is a synapse's size determined? How is synapse type and strength specified through adhesive and secreted trans-synaptic signaling? How do the redundant synapse-inducing pathways interact with each other? Given the rapidly emerging improvements of technologies, especially super-resolution microscopy and high-throughput genomics and proteomics, the synapse development field will likely rapidly evolve in the near future.

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