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Review

The molecular machinery of synaptic vesicle exocytosis

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Abstract. At the synapse, neurotransmitters are released via Ca^{2+}-triggered exocytotic fusion of synaptic vesicles with the presynaptic plasma membrane. Synaptic vesicle exocytosis seems to share many basic principles and homologous proteins with other membrane fusion events. Conserved components of the general fusion machinery that participate in synaptic vesicle exocytosis include soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), ATPase *N*-ethylmaleimide-sensitive factor, Munc18/nSec1, Rab3 GTPase, and the exocyst proteins. In addition, synaptic vesicle exocytosis uses a set of unique components, such as synaptotagmin, complexin, Munc13, and RIM, to meet the special needs of fast Ca^{2+}-triggered neurotransmitter release. This review summarizes present knowledge about the molecular mechanisms by which these components mediate and/or regulate synaptic vesicle exocytosis.

Key words. Secretion; exocytosis; SNARE; vesicular transport; neurotransmitter release; synaptic vesicle cycle.

Introduction

Regulated release of neurotransmitters mediates neuronal communication and underlies virtually all functions of the nervous system, from sensory perception to learning and memory. At the nerve terminal, synaptic vesicles cycle through a series of trafficking steps (fig. 1). Neurotransmitter-filled synaptic vesicles are docked at a specialized region of the presynaptic plasma membrane known as the active zone [1]. The docked vesicles then go through a maturation process called priming to become fusion competent [2]. In response to action potential-induced Ca^{2+} influx, primed vesicles undergo rapid exocytotic fusion to release neurotransmitters. Following exocytosis, synaptic vesicle membranes and protein constituents are retrieved from the plasma membrane by endocytosis, and locally recycled for future rounds of exocytosis [3, 4]. Over the past decade, remarkable progress has been made in our understanding of the molecular mechanisms governing synaptic vesicle trafficking and neurotransmitter release.

In this review, we provide an overview of current information concerning the molecular mechanisms of synaptic vesicle docking, priming, and fusion. We first summarize the role of conserved protein components of the general fusion machinery in mediating synaptic vesicle exocytosis, and then discuss several unique regulatory components that contribute additional layers of control required by fast Ca^{2+}-triggered neurotransmitter release.

Conserved components of the general fusion machinery in synaptic vesicle exocytosis

Fusion of a transport vesicle with its target membrane is a fundamental process essential to cellular organization and function of all eukaryotic cells. Recent progress has revealed that several protein families involved in fusion are conserved from yeast to human, and are shared not only by constitutive and regulated exocytosis but also by

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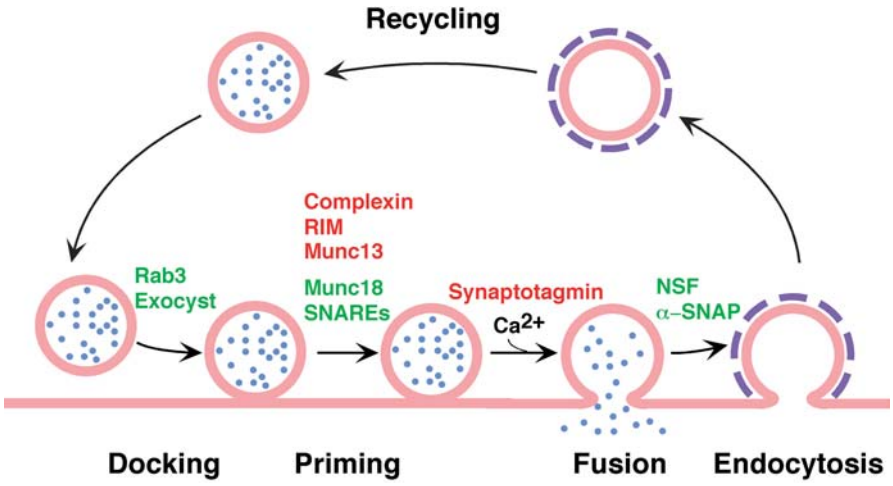

Figure 1. Protein components of the synaptic exocytotic machinery. The synaptic vesicle cycle at the nerve terminal involves vesicle docking, priming, fusion, endocytosis, and recycling (see text for details). Indicated are proteins that have been implicated in the docking, priming, and fusion steps. Conserved protein components of the general fusion machinery are shown in green, whereas unique components of synaptic vesicle exocytosis are shown in red.

various intracellular membrane fusion events [5–7]. The conserved protein families include soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), ATPase *N*-ethylmaleimide-sensitive factor (NSF), Munc18/Sec1, Rab GTPases, and protein components of the exocyst complex. This conservation suggests that virtually all membrane fusion processes, including synaptic vesicle exocytosis, use the same common molecular machinery for fusion.

SNAREs, the core of the membrane fusion machinery

The central players in all membrane fusion events seem to be SNAREs, a superfamily of membrane-associated proteins characterized by a ~60 amino-acid α-helical coiled-coil domain called the SNARE motif [5, 6, 8, 9]. These proteins were initially categorized as v-SNAREs and t-SNAREs based on their localization on vesicle or target membrane [5], and later reclassified as R-SNAREs and Q-SNAREs according to the conserved arginine or glutamine residue in the center of their SNARE motifs [10]. The human genome contains 36 SNAREs, of which 21 have yeast orthologues [6, 11]. These SNAREs are localized to distinct membrane compartments of the secretory and endocytic trafficking pathways, and contribute to the specificity of intracellular membrane fusion processes [6, 9, 12, 13].

Synaptic vesicle exocytosis requires three neuronal SNAREs: vesicle-associated membrane protein (VAMP2), also called synaptobrevin), syntaxin 1, and the 25-kDa synaptosomal-associated protein (SNAP-25). VAMP2 is an R-SNARE originally identified as an integral membrane protein of synaptic vesicles [14, 15]. Syntaxin 1 is a neuronal plasma membrane Q-SNARE first described as an antigen for a monoclonal antibody called HPC-1 [16, 17], and subsequently identified as a binding partner for synaptotagmin and the N-type calcium channel [18, 19]. SNAP-25 is another Q-SNARE initially identified as a brain-specific protein localized to neuronal plasma membrane via palmitoyl groups covalently attached to the cysteine residues [20]. VAMP2 and syntaxin 1 each contain a single SNARE motif adjacent to the carboxyl-terminal transmembrane domain, whereas SNAP-25 contains two SNARE motifs connected by a linker region bearing the palmitoylated cysteine residues [8]. The four SNARE motifs from these three proteins assemble into a parallel four-stranded helical bundle to form an extremely stable ternary complex called the SNARE complex [21, 22]. Interference with the integrity of such a superhelical structure by various mutations in neuronal SNAREs inhibits synaptic vesicle exocytosis [23–27]. Moreover, specific cleavage of neuronal SNAREs by clostridial neurotoxins prevents the assembly of a stable SNARE complex and blocks neurotransmitter release without affecting the docking of synaptic vesicles [28–30]. Targeted gene disruption of neuronal SNAREs in *Drosophila*, *Caenorhabditis elegans*, and mice abolishes action potential-evoked neurotransmitter release, further demonstrating an essential role for these proteins in Ca^{2+}-stimulated synaptic vesicle exocytosis [31–36].

Despite overwhelming evidence indicating the critical importance of neuronal SNAREs and the SNARE complex in synaptic vesicle exocytosis, their precise role in the fusion process remains controversial [9, 37, 38]. Formation of the SNARE complex in a *trans* configuration

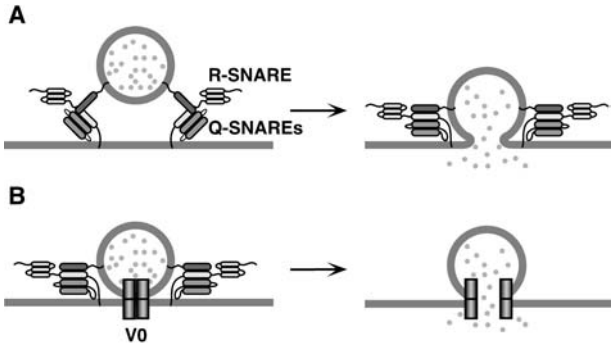

Figure 2. Two current models of membrane fusion. (*A*) SNARE-mediated fusion. Formation of *trans*-SNARE complexes between R-SNARE (VAMP) and Q-SNAREs (syntaxin 1 and SNAP-25) may occur by zippering towards the membrane-anchoring regions to pull the opposing membranes into close contact, which may drive membrane fusion [39, 40]. (*B*) V0 pore-mediated fusion. *Trans*-SNARE complex formation brings the two membranes together, allowing the assembly of V0 sectors of the vacuolar H^+-ATPase from the opposing membranes into *trans*-V0 complexes. The *trans*-V0 complexes in vacuole fusion also contain calmodulin and the Q-SNARE Vam3p (not shown) but not the R-SNARE Nyv1p. The two opposed V0 sectors may form a gap junction-like channel with a central pore, and radial opening of this proteinaceous pore could initiate fusion [56].

has been proposed to occur in a zipper-like fashion to bring synaptic vesicle and plasma membrane into juxtaposition, which may provide a driving force for membrane fusion (fig. 2A). Consistent with this 'zipper' model of fusion [39, 40], the assembly of the SNARE complex has been shown to serve as the minimal machinery for membrane fusion in reconstituted liposomes [41, 42]. In addition, a micromolar concentration of Ca^{2+} can trigger SNARE complex formation and concomitant fusion between purified synaptic vesicles and reconstituted Q-SNARE-containing liposomes [43]. Complete assembly of the SNARE complex also seems to correlate temporally with Ca^{2+}-stimulated fusion in permeabilized PC12 cells [44, 45], although the rate of exocytosis measured in this system is too slow to resolve the priming from the fusion reaction. Real-time measurement of exocytosis in adrenal chromaffin cells suggests that SNARE complexes exist in a dynamic equilibrium between a loose and a tight state, and the assembly of fully zippered SNARE complexes from these states is required for fast Ca^{2+}-triggered exocytosis [46, 47].

While the above results support a fusogenic role for SNARE complex assembly, other studies suggest that the SNARE complex might not participate in fusion per se, but acts, rather, at a step preceding the actual membrane fusion reaction. Recent evidence from experiments in synaptosomes and chromaffin cells indicates that the SNARE complex might be fully assembled at the priming step to regulate the formation of readily releasable vesicles [25, 48]. In addition, studies of cortical vesicle exocytosis in sea urchin eggs reveal that the SNARE complex assembly is not essential for the fusion reaction, but seems to promote the Ca^{2+} sensitivity of fusion [49–51]. A Ca^{2+}-sensing role of the SNARE complex is also supported by recent experiments in chromaffin cells [52]. In yeast vacuole fusion, *trans*-SNARE complex formation appears to occur at a step upstream of the fusion reaction [53]. The vacuole fusion involves several proteins downstream of SNARE complex formation, namely, calmodulin [54], protein phosphatase 1 [55], and the V0 sector of the vacuolar H^+-ATPase [56]. Two V0 sectors from the opposing membranes have been proposed to form a proteinaceous fusion pore to mediate membrane fusion (fig. 2B). This pore model of fusion [37, 56] seems consistent with the earlier reports suggesting that the V0 sector (also called the mediatophore) from *Torpedo* electric organ may mediate the release of the neurotransmitter acetylcholine [57, 58]. SNAREs have been found to associate with the V0 sector in the yeast vacuole [56] and in mammalian synaptosomes [59], raising the possibility that SNAREs might regulate the formation of a *trans*-V0 complex or opening of the V0 pore. However, unlike SNAREs whose mutations result in lethality because of impaired intracellular fusion processes, V0 mutants are able to survive on acidic media [56], arguing against an essential role for V0 in membrane fusion. Furthermore, the V0 pore model is inconsistent with the recent observation that the V0 subunits are not enriched at the fusion sites, i.e., the vertices of docked vacuoles [60].

Neuronal SNAREs interact with a number of other proteins [61], including Munc18/nSec1 [62], Munc13 [63], synaptotagmin [18, 64], complexin [65], tomosyn [66], Staring [67], SNIP [68], and Spring [69]. Munc18, Munc13, synaptotagmin, and complexin are perhaps the best-characterized SNARE-interacting proteins, and will be discussed later in this review. The roles of other SNARE-interacting proteins remain largely unknown, although some seem to regulate the assembly of SNARE complexes. A novel SNARE-interacting protein, Staring, has been shown to regulate the degradation of syntaxin 1 by acting as an E3 ubiquitin-protein ligase [67]. It will be important to find out whether SNAREs or other factors downstream of SNARE complex formation execute the fusion reaction, and how these essential components of the fusion machinery act in conjunction with other proteins to achieve the temporal and spatial specificity of neurotransmitter release.

NSF, a chaperone-like ATPase for disassembly of cis-SNARE complexes

NSF is a hexameric protein that belongs to the AAA (ATPases associated with various cellular activities) superfamily of chaperone-like ATPases [70–73]. NSF and

its yeast homologue Sec18p were independently identified as cytosolic proteins required for constitutive vesicular trafficking by mammalian cell-free transport assays [74, 75] and by yeast genetic studies [76]. Subsequently, NSF has been shown to function also in synaptic vesicle exocytosis [21, 23, 77–79]. NSF binds to the SNARE complex via SNAPs to form a larger 20S complex [80]. Hydrolysis of ATP by NSF then dissociates the 20S complex into individual components [21, 77]. Although NSF-mediated disassembly of the SNARE complex was initially thought to cause membrane fusion [5, 21, 77], this turns out not to be the case [6, 73]. NSF appears to dissociate only the *cis*-SNARE complex assembled on the same membrane, but not the trans-SNARE complex formed between opposing membranes in reconstitution experiments [81]. However, the yeast NSF homologue Sec18p, when present in excess, has been reported to disassemble the *trans*-SNARE complex formed between vacuolar membranes [53].

Based on biochemical studies, a likely function for NSF is to act at a post-fusion, pre-endocytosis step to disassemble *cis*-SNARE complexes accumulated in the plasma membrane as a result of vesicle fusion (fig. 3). Furthermore, NSF may also participate in the disassembly of *cis*-SNARE complexes recycled onto clathrin-coated vesicles [82] and synaptic vesicles [83]. Consistent with this model, temperature-sensitive mutations in *Drosophila* NSF result in excess accumulation of *cis*-SNARE complexes, leading to activity-dependent inhibition of synaptic vesicle exocytosis [23, 79, 84, 85]. Interestingly, the number of docked vesicles is significantly increased in *Drosophila* NSF mutants [79, 84], suggesting that in addition to a post-fusion role, NSF may mediate a post-docking, pre-fusion priming step to regulate the supply of readily releasable vesicles. A post-docking, priming role for NSF and its cofactor α-SNAP is also supported by evidence from studies in PC12 and chromaffin cells [86–89] and in squid giant synapses [78, 90]. The chaperone-like activity of NSF may be required for conferring and/or maintaining the fusion competence of SNAREs to allow the formation of *trans*-SNARE complexes.

Munc18/nSec1, a syntaxin 1-binding protein essential for fusion

In addition to SNAREs, another obligatory component of the general fusion machinery is Sec1/UNC-18, a conserved family of hydrophilic proteins with no recognizable domains or motifs [91, 92]. Sec1 and UNC-18 protein were originally identified by genetic screens in yeast [76, 93] and *C. elegans* [94, 95], respectively. In yeast, there are four Sec1-related proteins, each of which is required for specific intracellular membrane fusion steps [6, 11, 96]. The human genome contains seven Sec1/UNC-18 homologues [11], three of which, Munc18 (also named Munc18a, Munc18-1, nSec1, rbSec1), Munc18b (also called Munc18-2), and Munc18c (also called Munc18-3), are involved in exocytosis [62, 97–100]. Munc18 is highly enriched in neurons and has been shown to be absolutely required for synaptic vesicle exo-

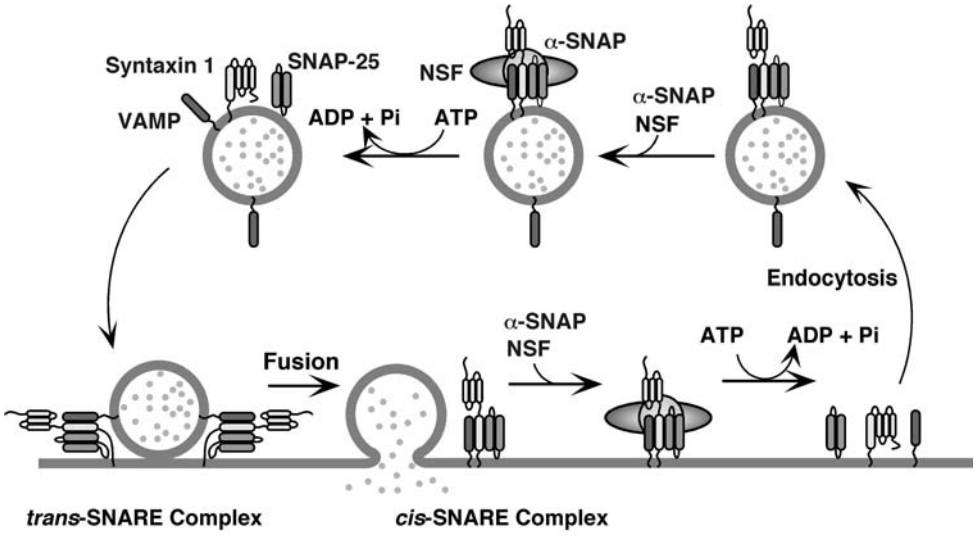

Figure 3. Current view of the role of NSF and its cofactor α-SNAP in synaptic vesicle trafficking. NSF/α-SNAP act after fusion to disassemble *cis*-SNARE complexes on the plasma membrane prior to endocytosis to recycle SNAREs for another round of fusion [85]. Some *cis*-SNARE complexes are recycled onto clathrin-coated vesicles [82] and synaptic vesicles [83], and these vesicular *cis*-SNARE complexes are subjected to NSF-mediated disassembly prior to docking. NSF/α-SNAP could also act after docking to dissociate any remaining *cis*-SNARE complexes in order to re-activate them for the formation of fusion-competent *trans*-SNARE complexes that are resistant to NSF-mediated disassembly [81].

cytosis by gene ablation studies in mice [101]. The essential role of UNC-18 in neurotransmitter release is also supported by genetic evidence from *C. elegans* and *Drosophila* [100, 102–104].

While Munc18 is clearly indispensable for synaptic vesicle exocytosis, the exact function of Munc18 in the fusion process remains unclear. Elimination of Munc18 expression in mice completely abolishes neurotransmitter release without affecting synaptic vesicle docking, suggesting that Munc18 functions at a post-docking step [101]. A post-docking role of Munc18 also receives support from microinjection experiments in squid giant synapse [105] and mutation studies in *Drosophila* [104]. Consistent with a post-docking role, a recent study in adrenal chromaffin cells suggests that Munc18 acts at a late stage in the fusion process, participating in the regulation of the fusion pore dynamics [106]. On the other hand, analysis of Munc18 null mutant chromaffin cells argues against such a late role of Munc18 in fusion, and suggests that Munc18 may participate in the docking of large dense-core vesicles to the plasma membrane [107].

The molecular mechanism by which Munc18 and its homologues mediate membrane fusion is not understood at present. In mammalian brain, Munc18 was independently discovered as a protein that binds strongly to neuronal Q-SNARE syntaxin 1 [62, 97, 98]. Biochemical and structural studies reveal that Munc18 interacts selectively with syntaxin 1 in the closed conformation (fig. 4A), a default configuration of syntaxin 1 that prevents its interaction with SNAP-25 and VAMP2 to form the SNARE complex [108–110]. Munc18 was initially thought to act as a negative regulator of membrane fusion by inhibiting SNARE complex assembly [103, 111]. However, this model does not seem to fit with the essential role of Munc18 in exocytosis as revealed by loss-of-function studies [101–103]. To accommodate a positive role of Munc18, later models proposed that Munc18 may have a chaperone-like function to regulate the transition of syntaxin 1 from the closed to open conformation, thus facilitating SNARE complex formation in conjunction with other regulatory proteins such as a Rab and/or Rab effector [109], tomosyn [66], or Munc13 [112] (see below).

Surprisingly, although Munc18 and its homologues play a conserved, essential role in various fusion reactions throughout evolution, these proteins do not share a common mode of interaction with corresponding syntaxins (fig. 4). In yeast, Sec1p does not bind directly to Sso1p, a plasma membrane syntaxin that adopts a closed conformation [113]. Instead, Sec1p binds to assembled SNARE complexes (fig. 4B) and seems to act downstream of *trans*-SNARE complex formation in promoting exocytosis [114, 115]. By comparison, the Sec1 homologue Sly1p binds to the yeast endoplasmic reticulum and Golgi syntaxins (Ufe1p and Sed5p) via a short, conserved N-terminal peptide motif (fig. 4C), which is fully compatible with SNARE complex formation [116]. Indeed, Sly1p-associated Sed5p allows efficient formation of SNARE complexes and contributes to the specificity of SNARE complex assembly [117]. A similar, N-terminal peptide motif-dependent binding was also observed between the Sec1 homologue Vps45p and the yeast trans-Golgi network/endosomal syntaxin Tlg2p [118]. In contrast, the Sec1 homologue Vps45p and Vps33p do not interact directly with the yeast prevacuolar and vacuolar syntaxins (Pep12p and Vam3p) but, rather, appear to function as part of novel multi-protein complexes to regulate SNARE complex formation in these compartments [60, 119–121]. The diverse interaction patterns of the Munc18/Sec1-related proteins could mean that these proteins operate in membrane fusion via a yet undiscovered common mechanism that is not related to their ability to bind syntaxins. Alternatively, they might function through distinct mechanisms to impose temporal or spatial specificity on different types of membrane fusion events. For example, the unique mode of interaction between Munc18 and syntaxin 1 may provide a means to ensure that synaptic vesicles do not fuse immediately after docking and yet can be induced to fuse rapidly upon activation. Munc18 also interacts with two novel proteins called mint [122] and Doc2 [123], but the functional significance of these interactions is currently unknown.

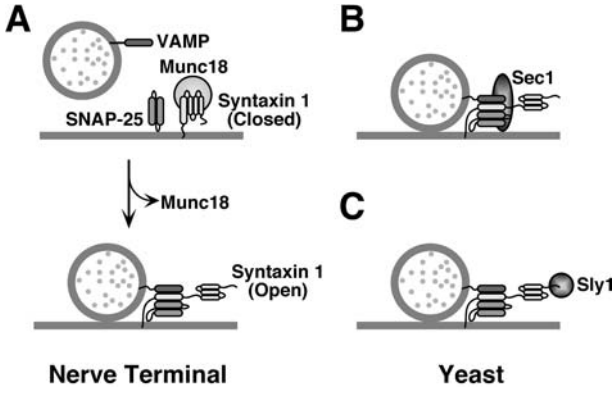

Figure 4. Synaptic vesicle exocytosis involves a unique mode of interaction between Munc18/nSec1 and syntaxin 1. (*A*) Munc18 binds specifically to the closed conformation of syntaxin 1, which prevents the formation of SNARE complexes [108–110]. (*B*) Yeast Sec1p binds to assembled SNARE complexes but not to free Sso1p, a plasma membrane syntaxin [114]. (*C*) The yeast Sec1 homologue Sly1p binds to an N-terminal peptide motif of Golgi syntaxin Sed5p [116], which allows efficient formation of SNARE complexes [117].

Rab3, a small GTPase that regulates vesicular trafficking

Rab proteins are members of the Ras superfamily of monomeric small GTPases that function in vesicular traf-

ficking steps throughout the secretory and endocytic pathways [124–126]. A role for Rab proteins (Sec4p and Ypt1p) in vesicular trafficking was first discovered in yeast by genetic screens [127, 128]. Subsequently, 11 Rab proteins have been identified in yeast and shown to associate with specific membrane compartments and regulate discrete intracellular trafficking events [129]. In humans, there are at least 60 Rab proteins, of which Rab3 has been implicated in regulated exocytosis of neurotransmitters and hormones [11, 125, 130]. Rab 3 has four isoforms, Rab3A, B, C, and D, which are differentially expressed in neuronal and endocrine tissues [6, 131, 132]. Rab3A is the most abundantly expressed isoform in brain where it is present in virtually all synapses, while Rab3B and Rab3C are only present in a subset of synapses [6, 133]. Like other Rab proteins, Rab3 interacts with membranes via C-terminal geranylgeranyl moieties, and cycles between a synaptic vesicle-associated, GTP-bound form and a cytosolic GDP-bound form [134, 135].

Recent progress in our understanding of Rab function reveals that a single Rab protein (e.g., Rab5) is capable of regulating multiple stages of vesicular trafficking, including vesicle budding, motility, docking, and fusion [125, 135]. Consistent with this common theme of diverse action, Rab3A has been implicated in the regulation of several stages of synaptic vesicle trafficking. First, Rab3A appears to be involved in regulating synaptic vesicle targeting to and docking at the active zone. Loss-of-function mutations in the only *C. elegans rab3* gene lead to a significant depletion of synaptic vesicles at presynaptic terminals and a concomitant elevation of vesicles along the axons [136]. In mice, although Rab3A deletion does not appear to affect synaptic vesicle distribution at the resting state, it abolishes the activity-dependent recruitment of synaptic vesicles to the active zone and impairs the replenishment of docked vesicles after exhaustive stimulation [131, 137]. Second, Rab3A seems to also act at a post-docking step to regulate synaptic vesicle fusion. In Rab3A null mutant mice, electrophysiological studies reveal that while the size of the readily releasable pool of vesicles is unaltered, Ca^{2+}-triggered synaptic vesicle exocytosis and paired pulse facilitation is increased [138], and mossy fiber long-term potentiation (LTP) is abolished [139]. Support for a role of Rab3A at a late step during Ca^{2+}-triggered exocytosis is also provided by studies in *Aplysia* neurons injected with a GTPase-deficient form of Rab3 [140]. Third, Rab3A might coordinately regulate the coupling between synaptic vesicle exocytosis and endocytosis through its putative effector Rabphilin [141, 142].

The diverse actions of Rab proteins are mediated via their multiple effectors, which usually interact with the GTP-bound form of Rabs [125, 135]. Rab3A has at least five potential effectors, called Rabphilin, RIM, Noc2, PRA1, and calmodulin. Rabphilin is a synaptic vesicle-associated, Rab3A-binding protein that has been suggested to function in both synaptic vesicle exocytosis and endocytosis [132, 134, 141–143]. However, targeted gene disruption of Rabphilin in mice and *C. elegans* did not lead to any of the phenotypes observed in Rab3A mutants, arguing against a role for Rabphilin as a downstream effector of Rab3A in synaptic vesicle trafficking [144, 145]. RIM is a Rab3-interacting molecule that specifically localized to the active zone [146]; its role in synaptic vesicle exocytosis will be discussed later in this review. Noc2 shares a homologous Rab3-binding domain with Rabphilin and RIM, and is expressed at very low levels in brain but highly enriched in endocrine tissues [147]. Thus, Noc2 probably does not play a significant role in synaptic vesicle exocytosis, but may mediate the effect of Rab3A in regulated exocytosis in endocrine cells [148]. PRA1 (prenylated Rab acceptor 1) is an evolutionarily conserved, ubiquitously expressed protein that binds to prenylated Rab3A and to several other prenylated small GTPases [149, 150]. PRA1 is localized to the Golgi complex and post-Golgi vesicles, and may participate in vesicle formation at the Golgi complex and help to recruit R-SNARE VAMP2 into the budding vesicles [149, 151]. In neurons, PRA1 has been shown to associate with synaptic vesicles and interact with Piccolo, an active-zone cytomatrix protein [1, 152], suggesting that PRA1 may mediate the effect of Rab3A in synaptic vesicle targeting to or docking at the active zone. Calmodulin is able to bind Rab3A and causes it to dissociate from the synaptic vesicle membrane in a Ca^{2+}-dependent manner [153]. Mutation studies in transfected PC12 cells suggest that the interaction of Rab3A with Ca^{2+}/calmodulin, but not with Rabphilin and RIM, may be involved in mediating the effect of Rab3A on Ca^{2+}-triggered exocytosis [154]. Further research is needed to determine whether these Rab3A-interacting proteins are physiologically important Rab3A effectors and how they are coordinated to mediate the diverse actions of Rab3A in synaptic vesicle trafficking and neurotransmitter release.

Exocyst, a multi-protein tethering complex in vesicle docking

Specific docking of a transport vesicle to its target membrane plays a crucial role in the membrane fusion process. The initial docking step (also known as tethering) in various fusion events is mediated by unique tethering factors that are often large multi-protein complexes [155, 156]. At the plasma membrane, vesicle docking involves a large tethering complex called the exocyst, which comprises eight proteins: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p (fig. 5). The exocyst was originally identified in yeast, where it is concentrated at the exocytotic fusion sites and plays an essential role in se-

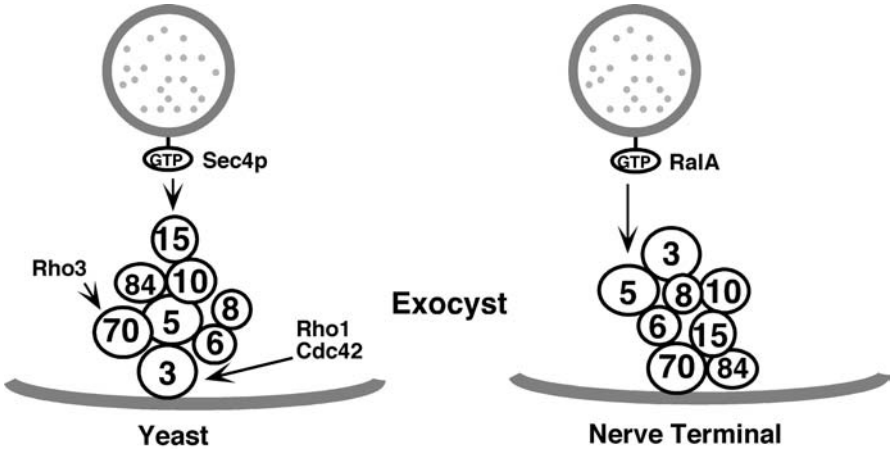

Figure 5. The exocyst is a conserved tethering factor in vesicle docking to the exocytotic fusion sites. The exocyst complex comprises eight proteins: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p. The organization of these proteins in the complex is depicted based on reported protein-protein interactions for yeast [160, 277] and mammalian [169] exocyst components. In yeast, Sec3p is the plasma membrane-proximal component that marks the exocytotic sites [158], whereas in mammalian cells, Exo70p seems to be the plasma membrane-proximal component [169]. The interactions between the exocyst and GTPases are indicated by arrows and discussed in the text. The interaction between the exocyst and vesicle-associated GTPases (Sec4p and RalA) may mediate initial docking of vesicles to the exocytotic fusion sites on the plasma membrane.

cretion [76, 157–160]. Yeast genetic studies suggest that the exocytst acts upstream of the SNARE complex assembly [115], but downstream of Sec4p, a Rab GTPase that is associated with secretory vesicles and required for exocytosis [127, 160, 161]. The yeast exocyst component Sec15p binds directly to the GTP-bound form of Sec4p, and this binding, together with the protein-protein interactions within the exocyst complex, has been proposed to mediate the initial docking of secretory vesicles to the exocytotic fusion sites [160]. In addition to Sec4p, members of the Rho family of small GTPases, namely, Rho1, Rho3, and Cdc42, have been shown to interact with the yeast exocyst components Sec3p and Exo70p [162–164]. These interactions may be involved in the coordination of exocytosis with polarized yeast cell growth.

The mammalian exocyst complex (also referred to as the sec6/8 complex) is ubiquitously present in all cells, and regulates vesicle targeting to the plasma membrane for exocytosis and polarized membrane insertion [165–170]. In developing neurons, the exocyst complex was found at high levels in regions of active neurite outgrowth and synaptogenesis prior to the appearance of synaptic vesicle clusters, and the exocyst levels appear to be greatly reduced in mature synapses [171]. Based on this expression pattern, the exocyst has been hypothesized to function primarily during neurite outgrowth and synaptogenesis, perhaps in specifying the exocytotic fusion sites and/or in targeting vesicles to these sites [167, 171]. Furthermore, a recent study suggests that the exocyst may promote neurite outgrowth by orienting microtubules toward specific domains on the plasma membrane [172].

In addition to a developmental role, the exocyst also seems to function in mature nerve terminals, as several groups have reported the presence of the exocyst at easily measurable levels in synaptosomes from adult animals [165, 173, 174]. The exocyst has been found to interact with the neuronal Q-SNARE syntaxin 1, suggesting possible involvement of the exocyst in synaptic vesicle exocytosis [165, 167, 171]. Interestingly, although there is so far no evidence connecting the mammalian exocyst to Rab or Rho proteins, the mammalian exocyst binds via Sec5p to the GTP-bound form of Ral, a small GTPase that is absent in yeast [174–176]. Ral has two isoforms, RalA and RalB, of which RalA is enriched in brain and associated with synaptic vesicles [177–179]. The exocyst is likely to be a downstream effector of RalA, since loss of Ral function inhibits the assembly of the exocyst complex [176] in a manner that is similar to the effect on yeast exocyst assembly of inactivating Sec4p [160]. Perturbations of RalA function or of the Ral-exocyst interaction inhibit vesicular transport of proteins to the basolateral surface in epithelial cells and block Ca^{2+}-dependent exocytosis from the neuroendocrine PC12 cells [176]. Furthermore, neuron-specific expression of a dominant-negative form of RalA in transgenic mice significantly inhibits the refilling of the readily releasable pool of synaptic vesicles [173]. Together, these findings suggest that the interaction between the mammalian exocyst and Ral might mediate or regulate the recruitment of synaptic vesicles to the exocytotic fusion sites [173, 174]. Although the exocyst seems to play a conserved role in vesicle tethering, subsequent docking reactions of synaptic vesicles at the active zone are likely to involve other proteins, such as the large, active-zone cytomatrix proteins bassoon [180], piccolo [152], and RIM (see below).

Unique regulatory components in synaptic vesicle exocytosis

Compared with other forms of membrane fusion, synaptic vesicle exocytosis is much more tightly regulated both in time and space. Temporally, Ca^{2+}-triggered secretion of neurotransmitters occurs in less than a millisecond after the arrival of action potential [181]. Spatially, synaptic vesicle exocytosis takes place only at the active zone, and nowhere else along the axonal membrane. Moreover, the efficacy of neurotransmitter release can be modulated over a wide range as a result of synaptic activity and experience, which is believed to be an important mechanism underlying learning and memory [182]. These special features of synaptic vesicle exocytosis require participation of unique regulatory components to provide additional levels of control over the general fusion machinery. Evidence accumulated over the last several years indicates that synaptic vesicle exocytosis utilizes several regulatory components, including synaptotagmin, complexin, Munc13, and RIM, which do not have homologues in yeast.

Synaptotagmin, a likely Ca^{2+} sensor for triggering synaptic vesicle fusion

Unlike constitutive exocytosis where vesicle fusion occurs without an external stimulus, neurotransmitter-filled synaptic vesicles accumulate at the pre-synaptic terminal and undergo rapid exocytotic fusion only when triggered by Ca^{2+} influx. The best-characterized candidate Ca^{2+} sensor in triggering neurotransmitter release is synaptotagmin (also called synaptotagmin 1), an integral membrane protein of synaptic vesicles with two Ca^{2+}-binding C2 domains [183–185]. Synaptotagmin was initially described as p65, a 65-kDa synaptic vesicle protein identified in a monoclonal antibody screen [186], and renamed after its cloning in 1990 [187]. To date, 13 different synaptotagmin isoforms have been reported in humans [184], and a recent database search suggests the existence of an additional 6 potential isoforms [188]. Compelling evidence from genetic studies in mice, *Drosophila*, and *C. elegans* as well as microinjection studies in squid giant synapses indicates that synaptotagmin plays an essential role in fast Ca^{2+}-triggered synaptic vesicle exocytosis, most likely by acting as a major Ca^{2+} sensor [189–198].

X-ray crystallography and nuclear magnetic resonance (NMR) studies reveal that both C2 domains (called C2A and C2B) of synaptotagmin share a similar structure of an eight-stranded β sandwich [199–202]. The C2A domain of synaptotagmin has been shown to bind three Ca^{2+} ions [201], whereas the C2B domain binds two Ca^{2+} ions [202]. Synaptotagmin binds via both C2 domains to phospholipids in a Ca^{2+}-dependent manner with half-maximal binding Ca^{2+} concentrations in the range of 5–20 μM [197, 202–204], which correspond very well with physiological Ca^{2+} levels for triggering neurotransmitter release at some synapses [205–207]. Furthermore, Ca^{2+} binding seems to cause the penetration of synaptotagmin C2 domains into lipid membranes with a fast on-rate [204, 208] compatible with rapid Ca^{2+}-triggered exocytosis [209, 210]. These binding properties suggest that synaptotagmin may function as a Ca^{2+}-sensitive, phospholipid-binding machine to promote synaptic vesicle fusion by pulling two membranes together or by helping to release tension in the metastable primed state (fig. 6E) [202, 208]. Supporting this model, a point mutation (R233Q) in the synaptotagmin C2A domain that decreases its ability to bind phospholipids in response to Ca^{2+} causes a parallel decrease in the release probability and Ca^{2+} sensitivity of synaptic vesicle exocytosis in synaptotagmin knockin mice [197]. However, mutations that disrupt the Ca^{2+}-binding sites in the synaptotagmin C2A domain did not affect Ca^{2+}-triggered neurotransmitter release in *Drosophila* [211]. The apparent lack of phenotype could be due to the redundancy of C2A and C2B domains in their Ca^{2+}-dependent phospholipid-binding activities [202, 212]. Indeed, mutations in the Ca^{2+}-binding sites of the *Drosophila* synaptotagmin C2B domain caused a dramatic (>95%) inhibition of Ca^{2+}-evoked neurotransmitter release and a large decrease in Ca^{2+} sensitivity of synaptic vesicle exocytosis [198].

In addition to phospholipids, synaptotagmin exhibits Ca^{2+}-dependent interactions with a variety of other molecules, including other synaptotagmins [213, 214], syntaxin 1 [18, 203], SNAP-25 [64, 215], SNARE complex [64, 196, 204], Ca^{2+} channels [216], SV2 [217], calmodulin [218], RIM [219, 220] (see below), and phosphoinositides [221]. Moreover, synaptotagmin also binds in a Ca^{2+}-independent manner to endocytosis-related proteins such as AP2 [222] and Stoned [223–225], and inositol phosphates [226]. Most of these interactions are mediated via the C2B domain of synaptotagmin. However, given the recent report that the recombinant synaptotagmin C2B domain contains tightly bound bacterial contaminants that seem to alter binding properties of the C2B [227], some of these interactions need to be interpreted with caution [184]. The functional significance of most of these interactions remains to be established. The interaction of synaptotagmin with SNAREs has received most attention because it provides an attractive mechanism for conferring Ca^{2+} regulation to the SNARE fusion machinery [183, 185, 215]. However, in a reconstituted liposome fusion system, synaptotagmin could not confer Ca^{2+} sensitivity to the SNARE-mediated fusion reaction [228]. A major challenge remains in proving unequivocally that synaptotagmin is indeed a Ca^{2+} sensor and in elucidating its precise mechanism of action in neurotransmitter release.

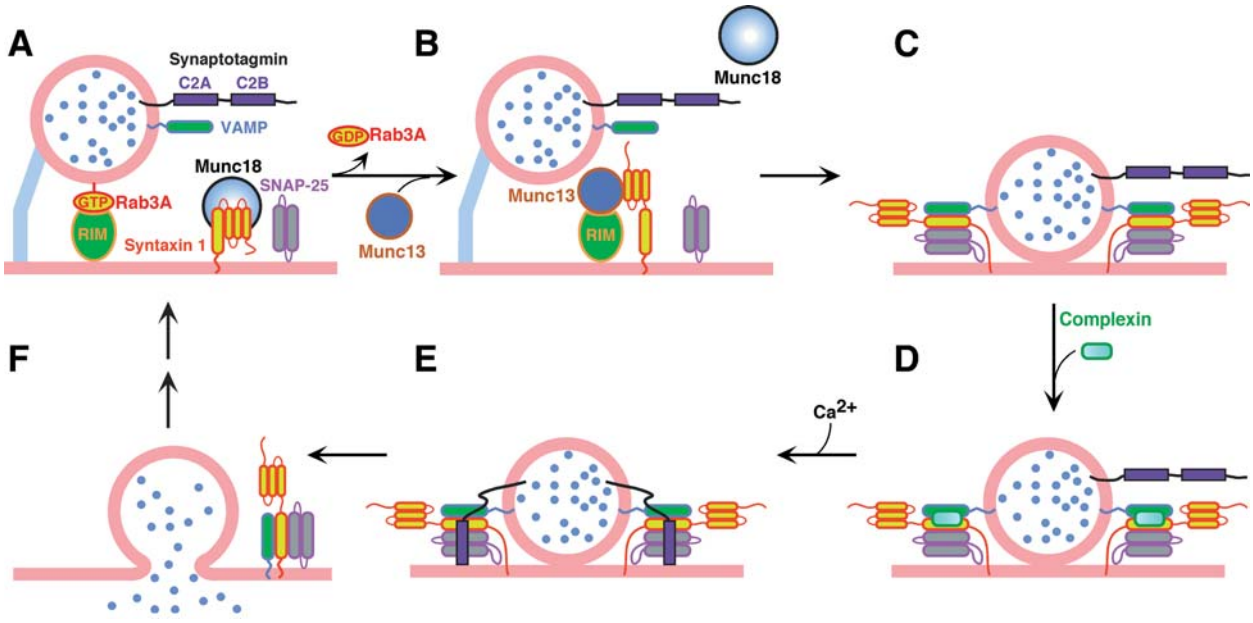

Figure 6. Molecular model of synaptic vesicle exocytosis. (*A*) Synaptic vesicle is docked to the active zone by an unknown mechanism (light blue bar) that may involve the exocyst and/or other proteins, such as piccolo and bassoon. The interaction between RIM and GTP-bound Rab3A may also contribute to the docking reaction. At this stage, Munc18 is associated with the closed conformation of syntaxin 1. (*B*) GTP hydrolysis causes the dissociation of Rab3A from RIM and the synaptic vesicle. RIM then binds Munc13 and activates the priming activity of Munc13 to displace Munc18 from syntaxin 1 and facilitate the conformational switch of syntaxin 1 into the open state. (*C*) Syntaxin 1 assembles with VAMP2 and SNAP-25 into the *trans*-SNARE complex, which pulls synaptic vesicle and plasma membrane into close contact. (*D*) Complexin binds to the fully assembled SNARE complex and stabilizes this metastable, fully primed state. (*E*) Ca^{2+} influx triggers the binding of synaptotagmin to the SNARE complex and penetration of synaptotagmin into the plasma membrane, leading to membrane fusion via a not yet understood mechanism. (*F*) After fusion, the *cis*-SNARE complex is dissociated by NSF/α-SNAP, and the SNAREs are recycled for another round of exocytosis.

Complexin, a SNARE complex-binding protein required for Ca^{2+}-triggered exocytosis

Complexin I and II (also called synaphin I and II) are two closely related ~15-kDa cytosolic proteins originally identified based on their brain-specific expression [229, 230] and ability to bind neuronal SNARE complexes [65]. Complexin I and II are widely expressed in brain where they are enriched in synapses [230–232]. Single-knockout mice lacking either complexin I or II exhibit only a mild phenotype, likely due to the functional redundancy of these two isoforms [233, 234]. Double-knockout mice lacking both complexins are perinatally lethal with a marked deficit in fast Ca^{2+}-triggered neurotransmitter release, suggesting that complexins positively regulate a late step of synaptic vesicle exocytosis [234]. Consistent with a late-acting positive role, perturbing the interaction between complexin and the SNARE complex by peptide injection into squid giant pre-synaptic terminals leads to a drastic reduction in neurotransmitter release at a post-docking step [235]. On the other hand, a late-acting inhibitory role has been suggested by microinjection studies in *Aplysia* neurons [231] and overexpression studies in PC12 [236] and adrenal chromaffin cells [237].

While complexins are clearly involved in synaptic vesicle exocytosis, their mechanism of action remains unclear. At least four models have been proposed to explain the potential role of complexins in synaptic vesicle exocytosis. First, complexins may act at a post-priming, Ca^{2+}-triggering step by either serving as a Ca^{2+} sensor, or working in conjunction with a Ca^{2+} sensor such as synaptotagmin, to increase the efficiency of the fusion reaction [234]. Supporting this model, analysis of complexin double-knockout mice reveals a post-priming defect in fast, synchronous Ca^{2+}-triggered release, which can be rescued completely by increasing the Ca^{2+} concentration [234]. Moreover, complexin and synaptotagmin are able to bind simultaneously to the assembled SNARE complex [65]. Second, complexins may promote oligomerization of *trans*-SNARE complexes for efficient, Ca^{2+}-regulated vesicle fusion [235]. Consistent with this possibility, studies in permeabilized PC12 cells suggest that fusion of a single vesicle is mediated by cooperation of three SNARE complexes [238], and complexin peptides that prevent SNARE complexes from oligomerizing block neurotransmitter release when injected into squid giant synapses [235]. Third, complexins may stabilize a metastable primed state with a fully assembled SNARE complex to ensure fast neurotransmitter release upon

Ca^{2+} influx (fig. 6D) [239]. Evidence supporting this model has been provided by biochemical and biophysical studies showing that complexin binds in anti-parallel fashion to the groove between synaptobrevin and syntaxin within the four-helix bundle of the SNARE complex [239–242]. The interaction between complexin and the SNARE complex has a fast on-rate [242], and seems to stabilize the interface between synaptobrevin and syntaxin helices in the SNARE complex, which might help to overcome the repulsive forces between the opposed membranes [239]. Fourth, complexins may function during or following membrane fusion to control the closure of the fusion pore, allowing the kiss-and-run recycling of exocytosed vesicles into the readily releasable pool [237]. This model is based on the observation that overexpression of wild-type complexin II, but not its synaptobrevin-binding site-specific mutant (R59H), leads to premature termination of exocytotic events without affecting the rate of fusion pore opening and expansion in adrenal chromaffin cells [237]. Further studies are required to distinguish these models and clarify the mechanism of action of complexins in synaptic vesicle exocytosis.

Munc13, a diacylglycerol-binding protein essential for synaptic vesicle priming

A crucial step in synaptic vesicle exocytosis is priming, which confers fusion competence to docked vesicles, enabling them to undergo rapid exocytosis upon Ca^{2+} influx [2]. Recent evidence indicates that synaptic vesicle priming requires the Munc13/UNC-13 family of proteins [243–245]. UNC-13 was originally identified in *C. elegans* genetic screens for uncoordinated mutants [94]. Munc13 proteins are mammalian UNC-13 homologues, which are encoded by three distinct genes: Munc13-1, Munc13-2, and Munc13-3 [244, 246]. These Munc13 isoforms are differentially expressed in brain, where they are specifically localized to the active zone [244, 246–248]. Knockout studies in mice reveal that Munc13-1 is essential for synaptic vesicle priming at 90% of glutamatergic synapses, whereas Munc13-2 is the priming factor for the remaining glutamatergic synapses [249, 250]. At GABAergic synapses, Munc13-1 and Munc13-2 are coexpressed and play a redundant role in vesicle priming [250]. The cerebellum-specific Munc13-3 isoform seems to be involved in vesicle priming at parallel fiber-Purkinje cell synapses [251]. Support for a critical role of UNC-13 in vesicle priming is also provided by loss-of-function mutation studies in *Drosophila* and *C. elegans* and by overexpression studies in chromaffin cells [252–254].

Munc13/UNC-13 proteins contain a C1 domain that binds endogenous second-messenger diacylglycerol and its analogue phorbol ester, and serve as high-affinity diacylglycerol/phorbol ester receptors in brain [255, 256]. Genetic studies in mice and *C. elegans* indicate that Munc13/UNC-13 proteins mediate the modulation of neurotransmitter release by phorbol ester and by G protein-coupled diacylglycerol second-messenger pathways [257–260]. Studies of Munc13-1 knockin mice expressing a diacylglycerol/phorbol ester-binding site-specific mutation (H567K) indicate that diacylglycerol/phorbol ester binding activates the priming activity of Munc13, leading to enhanced refilling of the readily releasable vesicle pool and increased release probability [260]. Electrophysiological analysis of Munc13-1 and Munc13-2 single- and double-knockout mice suggests that activity-dependent diacylglycerol-mediated Munc13 activation underlies a pre-synaptic form of short-term plasticity termed augmentation [261].

Munc13-1 interacts with the N-terminal coiled-coil domain of syntaxin 1 and this interaction appears to be compatible with SNARE complex assembly [63], suggesting that Munc13-1 binds to the open conformation of syntaxin 1. In *C. elegans*, UNC-13 is able to transiently interact with UNC-18 bound to the closed conformation of syntaxin and displace UNC-18 from syntaxin [112]. These results suggest that Munc13/UNC-13 may prime synaptic vesicles for fusion by promoting a conformational switch of syntaxin 1 from the closed to open state, thereby facilitating SNARE complex formation (fig. 6B). Consistent with this model, overexpression of a constitutively open form of syntaxin has been shown to rescue the *unc-13* loss-of-function mutant phenotype, demonstrating that the open form of syntaxin is able to bypass the requirement for UNC-13 in synaptic vesicle priming [262]. However, the rescue by the open form of syntaxin is inefficient, because the rescued animals exhibit significantly reduced Ca^{2+}-triggered synaptic vesicle exocytosis compared to wild-type controls. Incomplete rescue by the open form of syntaxin suggests that UNC-13/Munc13 may play additional role(s) in synaptic vesicle exocytosis beside promoting the conformational switch of syntaxin 1 to the open state. Munc13 interacts with at least five other proteins, namely RIM [263] (see below), calmodulin [264], Doc2 [265, 266], an ARF6 exchange factor msec7-1 [267], and a brain-specific spectrin isoform β-spIIIΣ1 [268]. Whether and how these interactions regulate synaptic vesicle exocytosis remains to be determined.

RIM, an active-zone scaffolding protein for coordinating synaptic vesicle docking, priming and fusion

RIM (also called RIM1) was originally identified in a yeast two-hybrid screen as a 180-kDa, active zone-spe-

cific protein that binds specifically to the GTP-bound form of Rab3 [146]. Mammalian brain contains a second RIM protein, RIM2, which is also expressed in endocrine tissues [269, 270]. *C. elegans* contains only one RIM protein [271], which is encoded by *unc-10*, a gene first identified in the classic uncoordination mutant screen [94]. *C. elegans unc-10* mutants lacking RIM exhibit fivefold reduced levels of fusion-competent vesicles despite normal levels of docked vesicles, and this deficit can be rescued by overexpression of a constitutively open form of syntaxin, suggesting that Rim has a post-docking role in regulating synaptic vesicle priming [271]. Consistent with a post-docking role, elimination of RIM1 expression in mice significantly reduces neurotransmitter release probability without affecting synaptic vesicle docking [220]. In RIM1 knockout mice, short-term synaptic plasticity, such as paired pulse facilitation, paired pulse depression, and post-tetanic potentiation, is altered [220], and mossy fiber long-term potentiation is abolished [272]. The phenotypes of RIM mutant mice and *C. elegans* are broader and more severe than Rab3 mutants, suggesting that the function of RIM goes beyond being just a Rab3 effector [220, 271]. The RIM mutant phenotypes are much weaker than Munc13-1 and UNC-13 mutants, suggesting that RIM has a regulatory instead of an essential, executing role in synaptic vesicle exocytosis.

RIM1 knockout mice exhibited a selective, 60% decrease in expression levels of Munc13-1, but not Munc13-2 or other presynaptic proteins, suggesting that RIM1 normally associates with Munc13-1 [220]. Indeed, RIM1 has been shown to bind Munc13-1 but not Munc13-2 [263, 273]. The RIM1-Munc13-1 interaction seems to be important in regulating synaptic vesicle priming, as disruption of this interaction by overexpressing a RIM1-binding fragment of Munc13-1 resulted in a drastic reduction in the size of the readily releasable vesicle pool [263]. Interestingly, Munc13-1 and Rab3A bind to the same site in RIM1, and the binding of Munc13-1 and Rab3A to RIM1 is mutually exclusive [263]. This raises the possibility that RIM may coordinate synaptic vesicle docking and priming by sensing the arrival of Rab3A-containing vesicles through its interaction with Rab3A-GTP and then, after GTP hydrolysis, to activate the priming reaction via its interaction with Munc13-1 (fig. 6A, B).

In addition to Rab3 and Munc13-1, RIM1 also interacts with SNAP-25 [219], synaptotagmin [219, 220], Ca^{2+} channels [219], RIM-BPs [270, 274], cAMP-GEFII [269], and liprins [220]. As discussed earlier, SNAP-25 is an essential component of the synaptic vesicle fusion machinery, and synaptotagmin is a putative Ca^{2+} sensor for triggering synaptic vesicle exocytosis in response to Ca^{2+} influx. RIM-BPs (also called RBPs) are a family of RIM-binding proteins that also interact with presynaptic Ca^{2+} channels [270, 274]. cAMP-GEFII (also called cAMP sensor or Epac) is a cAMP-binding protein that is thought to be a direct target of cAMP in regulating exocytosis through its interaction with RIM [269]. Liprins are a family of LAR receptor tyrosine phosphatase-interacting proteins required for proper formation and function of the presynaptic active zone [275, 276]. The RIM1-liprin interaction may serve to anchor RIM1 at the active zone and/or mediate the effect of the LAR/liprin pathway on synaptic vesicle exocytosis. The interaction of RIM1 with multiple, key components of the neurotransmitter release machinery suggests that RIM1 may play a critical role in organizing a multi-protein presynaptic scaffold to coordinate vesicle docking, priming, and fusion reactions, thereby ensuring the temporal and spatial specificity and efficiency of synaptic vesicle exocytosis.

Concluding remarks

The realization that neurotransmitter release uses the same general fusion machinery as other intracellular membrane fusion events has catalyzed progress toward a mechanistic understanding of synaptic vesicle exocytosis. Conserved components of the fusion machinery, such as SNAREs, Munc18/nSec1, and Rab3, are critically involved in synaptic vesicle exocytosis. However, a major challenge remains in defining the exact action of these proteins and resolving the molecular mechanism underlying the fusion reaction. In addition, it remains to be determined whether the exocyst complex plays a role in synaptic vesicle docking, and what other proteins participate in mediating specific docking of synaptic vesicles to the active zone. Recent progress has revealed that synaptic vesicle exocytosis requires several unique components, including synaptotagmin, Munc13, RIM, and complexin. Future studies are needed to determine the precise role of these proteins and elucidate the biochemical basis of synaptic vesicle priming and Ca^{2+}-triggering reactions. Each of the above-mentioned exocytotic components also interacts with several other proteins, and determining the physiological relevance of these protein-protein interactions will be important. Furthermore, it will be crucial to understand how these exocytotic components are spatially organized and temporally coordinated to achieve the extraordinary speed, precision, and plasticity of neurotransmission. Synaptic vesicle exocytosis has fascinated neuroscientists and cell biologists for over half a century and will continue to be an exciting area of active research for the years to come.

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