Controversies in synaptic vesicle exocytosis

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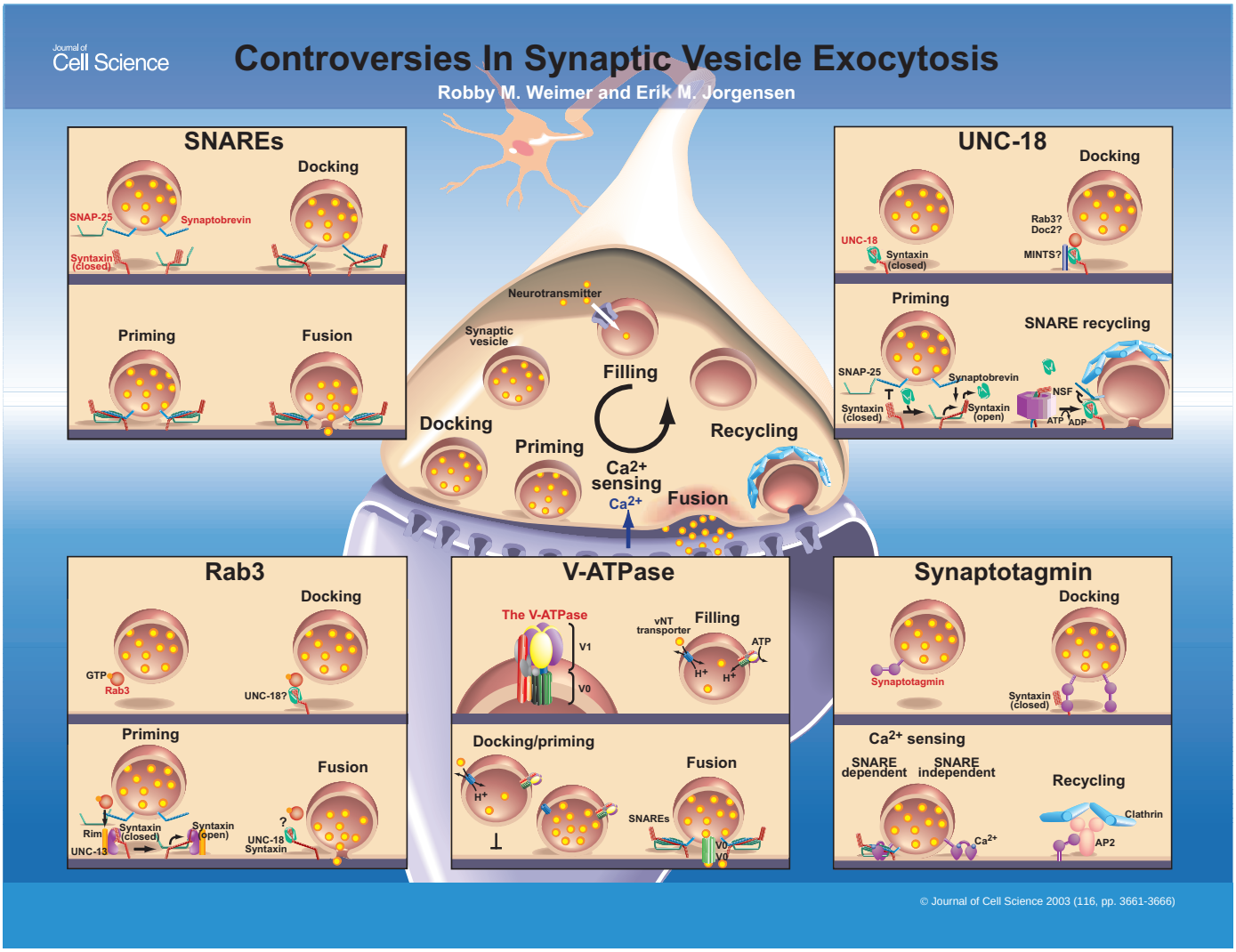
At the heart of synaptic transmission resides the synaptic vesicle cycle – a membrane trafficking pathway in which small membrane-bound vesicles mediate the release of neurotransmitter from presynaptic terminals. The cycle resembles general membrane trafficking and has three phases: vesicle filling, release and recycling. During filling, neurotransmitter is loaded into vesicles via vesicular neurotransmitter transporters. It is then released by exocytosis: vesicles dock with the plasma membrane and undergo a maturation step, termed priming; then, following influx of calcium through voltage-gated channels, a calcium sensor promotes fusion of the vesicle with the plasma membrane. Membrane fusion consumes vesicle membrane and vesicle proteins; thus, these components must be recycled to sustain neurotransmitter release.

The synaptic vesicle cycle is driven by members of protein families required for general membrane trafficking, including SNAREs, UNC-18, Rab3 and vacuolar H^+ ATPases (V-ATPases), and unique proteins, such as synaptotagmin, which regulate aspects of exocytosis unique to synapses. Although extensively studied, the precise roles of these proteins remain controversial. Here we survey current models proposed for their function in synaptic vesicle cycling.

SNAREs

SNARE (soluble NSF attachment protein receptor) proteins are ~18-32 kDa membrane-associated proteins that interact to form SNARE complexes (reviewed by Chen and Scheller, 2001). The SNARE complex at the synapse contains a bundle of four α-helices in which one helix is donated by the vesicle SNARE (v-SNARE, aka R-SNARE) synaptobrevin and three are donated by the target SNAREs (t-SNAREs, aka Q-SNAREs) SNAP-25 and syntaxin



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(Bennett and Scheller, 1993; Sollner et al., 1993b; Hanson et al., 1997; Sutton et al., 1998; Lonart and Sudhof, 2000). Inhibition of SNARE complex formation blocks neurotransmitter release (Littleton et al., 1998; Chen et al., 2001), thus the SNARE proteins are thought to promote synaptic vesicle exocytosis via SNARE complex formation.

Docking

SNARE complex formation was originally thought to dock synaptic vesicles to the plasma membrane (Sollner et al., 1993b). SNAREs localize to distinct subcellular compartments, representing unique molecular tags. Thus, formation of complexes between pathway-specific SNAREs might ensure that vesicles are targeted to the correct compartment. However, subsequent biochemical and genetic studies have failed to support such a role. In *Drosophila*, SNAREs can be promiscuous, the non-synaptic t-SNARE SNAP-24 can interact with syntaxin and synaptobrevin to form SNARE complexes in vitro (Niemeyer and Schwarz, 2000) and substitute for SNAP-25 in vivo (Vilinsky et al., 2002); SNARE interactions alone thus seem incapable of providing specificity for membrane targeting. Furthermore, perturbations of the SNAREs in squid and *Drosophila* fail to block the docking of synaptic vesicles (Hunt et al., 1994; Broadie et al., 1995).

Priming

Structural data indicate that SNARE complex formation in trans would bring the synaptic vesicle membrane into close proximity to the plasma membrane (Baumert et al., 1989; Bennett et al., 1992; Hanson et al., 1997; Lin and Scheller, 1997; Sutton et al., 1998), which might be essential for their fusion. Consistent with this notion, the size of the readily releasable pool (presumably primed vesicles) correlates with the level of SNARE complex assembly in vivo (Lonart and Sudhof, 2000).

Fusion

The current dogma is that SNARE complex formation is sufficient for the fusion step. Specifically, zippering together of the SNARE complex in trans provides the energy needed to drive membrane fusion. Consistent with this model, purified membranes containing only cognate SNARE proteins undergo membrane fusion (Weber et al., 1998; McNew et al., 1999; McNew et al., 2000). However, the rates observed are low (Weber et al., 1998), suggesting that other components may be required in vivo to support the rapid fusion rates observed at the synapse.

UNC-18

UNC-18 is a member of the Sec1p family: cytoplasmic proteins that bind to pathway-specific SNAREs (Jahn, 2000). *C. elegans* UNC-18 and its *Drosophila* and mammalian homologs ROP and Munc18-1 bind to the t-SNARE syntaxin with nanomolar affinity (Hata et al., 1993; Pevsner et al., 1994b; Ogawa et al., 1996; Matos et al., 2000). Thus, current models of its function incorporate this interaction.

Docking

In the absence of Munc18-1, dense-core vesicles in chromaffin cells fail to dock with the plasma membrane (Voets et al., 2001). UNC-18 physically interacts with the synaptic vesicle protein Doc2 (Verhage et al., 1997) and, in yeast, complexes containing its homolog Vps33p interact with the Rab3 homolog Ypt7p (Haas et al., 1995; Rieder and Emr, 1997; Ungermann et al., 1998; Eitzen et al., 2000; Price et al., 2000; Seals et al., 2000). Thus, UNC-18 may anchor synaptic vesicles to the plasma membrane by binding Doc2 or Rab3 on the vesicle and syntaxin on the plasma membrane. However, perturbation studies have not revealed a role for syntaxin in docking (see above). Alternatively, UNC-18 could facilitate docking through interactions with other membrane-associated proteins, such as the Mint-Cask-Neurexin complex (Okamoto and Sudhof, 1997; Biederer and Sudhof, 2000).

Priming

Although syntaxin must interact with synaptobrevin to prime vesicles for release, in solution syntaxin adopts a closed conformation; its N-terminus folds over and occludes the SNARE-binding domain (Calakos et al., 1994; Dulubova et al., 1999). Thus, syntaxin needs to be ‘opened’ for priming to occur. UNC-18 binds specifically to the closed configuration of syntaxin (Dulubova et al., 1999; Misura et al., 2000; Yang et al., 2000). Perhaps UNC-18 promotes exocytosis by opening syntaxin. Consistent with this model, expression of a truncated form of the syntaxin homolog in yeast, Tlg2p, partially suppresses mutations in the UNC-18 homolog Vps45p (Bryant and James, 2001).

Fusion

UNC-18 could also function in fusion. First, UNC-18 binds to syntaxin, a putative fusion mediator. Second, in Munc18-1-null mice, fusion events are undetectable at central synapses (Verhage et al., 2000). Third, overexpression of altered Munc18-1 protein in chromaffin cells alters the dynamics of the fusion pore in single-granule exocytosis (Fisher et al., 2001); however, overexpression of wild-type Munc18-1 had no effect on fusion. How UNC-18 might regulate fusion remains elusive (see below).

SNARE recycling

Overexpression of UNC-18 in *Drosophila* inhibits neurotransmitter release in a syntaxin-dependent manner (Schulze et al., 1994; Wu et al., 1998; Wu et al., 2001), which suggests that UNC-18-syntaxin interactions inhibit rather than promote exocytosis. Although originally proposed to regulate the priming step, a possible role for the UNC-18 inhibitory activity is to retain syntaxin in the plasma membrane during recycling of vesicle components. Upon fusion, trans-SNARE complexes are converted into cis-SNARE complexes, which are disassembled by the ATPase NSF (N-ethylmaleimide-sensitive factor) (Wilson et al., 1992; Sollner et al., 1993a; Littleton et al., 1998). After disassembly, UNC-18 binding to ‘closed’ syntaxin might inhibit errant cis-SNARE complex formation before synaptobrevin is removed from the plasma membrane. Consistent with this hypothesis, UNC-18 is capable of inhibiting SNARE complex formation in

vitro (Pevsner et al., 1994a) and syntaxin levels are reduced in Munc18-1-null mice (Voets et al., 2001), which might be due to degradation of aberrant cis-SNARE complexes.

Rab3

Rabs are small GTPases that are associated with trafficking membranes (reviewed by Zerial and McBride, 2001), and essential for all membrane trafficking in yeast (reviewed by Lazar et al., 1997). The presynaptic Rab, Rab3, associates with synaptic vesicles (Fischer von Mollard et al., 1990; Nonet et al., 1997), and mutation of Rab3 alters neurotransmitter release (Geppert et al., 1994a; Nonet et al., 1997; Leenders et al., 2001). So, what is its role at the synapse?

Docking

Synaptic vesicles fail to cluster near release sites in *C. elegans* and mouse Rab3 mutants (Nonet et al., 1997; Leenders et al., 2001), and overexpression of Rab3 in PC12 cells increases the number of docked granules (Martelli et al., 2000). The yeast Rab3 homolog Ypt7p interacts with a complex containing the UNC-18 homolog Vps33p, and another yeast Rab3 homolog, Ypt1p, interacts genetically with the UNC-18 homolog Sly1p (certain Sly1 mutations bypass loss of Ypt1p) (Ossig et al., 1991). Thus, Rab3 might act via UNC-18 to promote docking.

Priming

Rab3 interacts with several synaptic proteins, including the active zone protein Rim (Rab3-interacting molecule) (Wang et al., 1997), which in turn interacts with UNC-13 (Betz et al., 2001) – a syntaxin-binding protein (Betz et al., 1997). Both Rim and UNC-13 are required for the priming of synaptic vesicles for exocytosis in mice (Brose et al., 2000; Schoch et al., 2002; Varoqueaux et al., 2002), *Drosophila* (Aravamudan et al., 1999) and *C. elegans* (Koushika et al., 2001; Richmond et al., 1999). Moreover, UNC-13 and Rim appear to function at the priming step to promote or stabilize the open state of syntaxin (Koushika et al., 2001; Richmond et al., 2001). Rab3 may play a role in this priming pathway by signaling the arrival of a docked synaptic vesicle to the Rim-UNC-13 priming complex.

Fusion

Rab3A-null mice exhibit high levels of neurotransmitter release during neuronal excitation even though the size of the readily releasable pool of vesicles and the calcium-dependence of release is unaffected (Geppert et al., 1994a). Rab3A may thus act as an inhibitor of fusion, perhaps to coordinate release. Consistent with such a role, overexpression of Rab3A in PC12 cells causes constitutive fusion of secretory vesicles (Schluter et al., 2002). However, the molecular mechanism by which Rab3 may regulate fusion is obscure. Perhaps, as the yeast genetic data suggest, Rab3 acts via UNC-18. Such an interaction could link Rab3 signaling to the fusion machinery, specifically syntaxin.

Nothing at all

An ugly fact confronting those trying to develop models for Rab3 function is that elimination of Rab3 has an extremely mild phenotype in both mice and worms (Geppert et al., 1994a; Nonet et al., 1997). We might have to confront the possibility that the important functions supplied by Rab proteins in other fusion events have been appropriated by other proteins in synaptic vesicle fusion. Rab3 may be a vestigial component of the synaptic vesicle cycle.

V-ATPase

The V-ATPase is a large macromolecule that uses ATP hydrolysis to pump protons into the synaptic vesicle to acidify this compartment. The V-ATPase consists of a cytoplasmic V1 complex that is attached to the transmembrane V0 complex (reviewed by Nishi and Forgac, 2002).

Loading

Neurotransmitters are concentrated into synaptic vesicles by transporters that use the electrochemical gradient between the vesicle lumen and the cytoplasm to drive neurotransmitter loading (reviewed by Schuldiner et al., 1995). Inhibition of V-ATPase activity in vitro blocks neurotransmitter transport into vesicles (Anderson et al., 1982; Hell et al., 1990; Moriyama et al., 1990). Thus, V-ATPase activity is probably required in vivo to generate the driving force for vesicle loading.

Docking/priming

Neurotransmitter release is quantal: the amount of neurotransmitter released in every fusion event is constant. Thus, there might be a mechanism to prevent the fusion of partially filled vesicles. In yeast, V-ATPase activity is required for the formation of trans-SNARE complexes and thus membrane fusion (Ungermann et al., 1999). A similar requirement at the synapse might provide a checkpoint to ensure that only vesicles fully loaded with neurotransmitter are docked or primed. However, blocking of the V-ATPase by bafilomycin does not block synaptic vesicle release suggesting that such a checkpoint may not exist (Zhou et al., 2000).

Fusion

Recent data suggest that vesicle acidification may not be the only function for the V-ATPase in synaptic vesicle exocytosis. Specifically, trans-V0 complexes assemble in a SNARE-dependent manner between yeast vacuolar membranes destined to fuse, which suggests a late role for the V0 complex in membrane fusion (Peters et al., 2001). Such a complex could catalyze lipid mixing during fusion or act as an aqueous pore for the release of neurotransmitter without the complete mixing of vesicular and plasma membranes.

Synaptotagmin

Synaptotagmin is a vesicle-associated protein that contains two C2 calcium-binding domains (reviewed by Chapman, 2002). Mutation of synaptotagmin blocks neurotransmitter release (Littleton et al., 1993; Geppert et al., 1994b). However, synaptotagmins are not found in all eukaryotes, which suggests that synaptotagmin is not a

component of the core membrane trafficking machinery but is rather an accessory protein which may regulate neurotransmitter release.

Docking

Synapses in *Drosophila* lacking synaptotagmin contain fewer morphologically docked synaptic vesicles per release site (Reist et al., 1998), which suggests that synaptotagmin promotes vesicle docking. Synaptotagmin binds syntaxin (Chapman et al., 1995), SNAP-25 (Schiavo et al., 1997), calcium channels (Kim and Catterall, 1997; Sheng et al., 1997) and lipids (Brose et al., 1992; Davletov and Sudhof, 1993; Zhang et al., 1998); perhaps these interactions link vesicles to the plasma membrane during docking. Alternatively, the reduced number of docked vesicles in *Drosophila* synaptotagmin mutants could be a secondary phenotype due to a requirement for synaptotagmin in vesicle recycling (see below).

Calcium sensing

Synaptotagmin binds calcium via its C2 domains with an affinity that corresponds to the calcium threshold for synaptic vesicle exocytosis (Brose et al., 1992; Heidelberger et al., 1994; Davis et al., 1999). Synaptotagmin might therefore function as the calcium sensor. Consistent with this hypothesis, synaptotagimin-I-knockout mice lack the fast calcium-dependent phase of neurotransmitter release even though wild-type levels of vesicles are docked at the membrane (Geppert et al., 1994b). Calcium binding to synaptotagmin might affect the SNARE complex, either positively or negatively, since synaptotagmin binds the SNARE complexes near the transmembrane domains of syntaxin and synaptobrevin (Brose et al., 1992; Ernst and Brunger, 2003; Kee and Scheller, 1996; Pevsner et al., 1994a; Schiavo et al., 1997; Wu et al., 1999). Alternatively, recent data suggest that synaptotagmin functions as a calcium sensor independently of SNARE complex interactions (Shin et al., 2003). Because synaptotagmin binds to lipids in a calcium-dependent manner (Brose et al., 1992; Davletov and Sudhof, 1993; Zhang et al., 1998), it might directly promote vesicle fusion.

Recycling

Synaptotagmin also seems to be required for the recycling of synaptic vesicle components. Ultrastructural analysis of synaptotagmin-null mutants in *Drosophila* and *C. elegans* indicate that their synaptic varicosities are depleted of synaptic vesicles (Jorgensen et al., 1995; Reist et al., 1998; Littleton et al., 2001) – a phenotype characteristic of a defect in endocytosis. In addition, synaptotagmin is known to bind proteins involved in clathrin-mediated endocytosis, specifically the α and μ subunits of the clathrin adaptor complex AP-2 (Zhang et al., 1994; Haucke and De Camilli, 1999; Haucke et al., 2000). Thus, synaptotagmin may play dual roles to both promote synaptic vesicle exocytosis and endocytosis.

Future directions

Biochemical and genetic approaches over the past 30 years have yielded an extensive catalog of proteins and protein interactions central to membrane trafficking. There is no shortage of models for how these interactions could account for the physiology of the synapse. In the future, convincing tests for these models must be designed and executed. The design of these experiments is likely to arise from the excellent data emerging from structural analyses of synaptic proteins. For example, NMR studies revealed that the t-SNARE syntaxin adopts two conformations – closed and open (Dulubova et al., 1999) – thus leading to the hypothesis that a presynaptic protein opens syntaxin to permit vesicle exocytosis. Such a factor would be expected to bind to syntaxin, and defects caused by the loss of this protein should be bypassed by the open form of syntaxin. These predictions were confirmed for the synaptic protein UNC-13. UNC-13 binds to syntaxin (Betz et al., 2001) and a constitutively open form of syntaxin bypasses the requirement for UNC-13 (Richmond et al., 2001). Elsewhere, researchers are taking advantage of structural data to engineer variants to test the relevance of the interactions between synaptotagmin and SNAREs, calcium and lipids during exocytosis (Fernandez-Chacon et al., 2001; Mackler et al., 2002; Robinson et al., 2002; Yoshihara and Littleton, 2002; Shin et al., 2003). Testing the importance of each of the proposed molecular interactions in this pathway will be critical if we are to develop a comprehensive model describing the molecular mechanism underlying synaptic vesicle exocytosis.

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