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REVIEW

THE SYNAPTIC VESICLE CLUSTER: A SOURCE OF ENDOCYTIC PROTEINS DURING NEUROTRANSMITTER RELEASE

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Abstract—Over the past few years significant progress has been achieved in understanding the molecular steps underlying the fusion and recycling of vesicles at central synapses. It still remains unclear, however, how the fusion event is linked with vesicle membrane retrieval. Several factors promoting the transition from exo- to endocytosis have been extensively studied, including levels of intracellular Ca^{2+}, the synaptic proteins involved at both sides of the vesicle cycle, posttranslational modification of endocytic proteins, and the lipid composition of recycled membranes. Recent studies in glutamate synapses indicate that vesicle clusters accumulated at the sites of synaptic contacts have a more complex organization than has previously been thought. Many endocytic proteins reside in the vesicle pool at rest and undergo cycles of migration between the active and periactive zones during synaptic activity. We propose that the local migration of endocytic proteins triggered by Ca^{2+} influx into the nerve terminal functions as one of the molecular mechanisms coupling exo- and endocytosis in synapses.

Key words: synapse, synaptic vesicles, calcium, active zone, periactive zone, endocytosis.

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Synapses are specialized signaling units composed of pre- and postsynaptic compartments. The presynaptic nerve terminal contains neurotransmitter-filled synaptic vesicles clustered at specialized regions of the presynaptic membrane, the active zones. Neurotransmitters are released at the active zone into the cleft separating the pre- and postsynaptic compartments upon fusion of synaptic vesicles (Murthy and De Camilli, 2003; Südhof, 2004). This fusion event is followed by vesicle membrane retrieval. Two major mechanisms have been implicated in this process: "kiss and run" and compensatory clathrin-mediated endocytosis (He and Wu, 2007; Jung and Haucke, 2007; Rizzoli and Jahn, 2007). In addition, different forms of bulk membrane retrieval can be recruited in central synapses during intense stimulation (Wu and Wu, 2007; Xu et al., 2008). The "kiss and run" mechanism presumably functions at the active zone, while clathrin-mediated endocytosis and possibly also bulk endocytosis occur at the periactive zone surrounding the sites of release. Several recent reviews have discussed the molecular basis of synaptic vesicle fusion and endocytosis (Becherer and Rettig, 2006; Jung and Haucke, 2007; Murthy and De Camilli, 2003; Sudhof, 2004). This review is focused on the mechanisms that link these two intimately coupled events in the central synapse.

ROLE OF Ca^{2+} INFLUX

It is well established that Ca^{2+} influx through voltage-gated Ca^{2+} channels, following arrival of action potentials to the nerve terminal, serves as a trigger for synaptic vesicle fusion with the plasma membrane (Chapman, 2002; Neher, 1998; Schneggenburger and Neher, 2005). There is now compelling evidence that this calcium influx also plays an essential role in synaptic vesicle endocytosis. The first clues for a role of Ca^{2+} for triggering endocytosis in nerve terminals came from experiments in *Drosophila* larval neuromuscular junctions. Uptake and release of the styryl dye Fei Mao styryl dye 1-43 (FM1-43) was used to monitor synaptic vesicle exocytosis. FM1-43 was found to specifically label subsynaptic domains in nerve terminals enriched in synaptotagmin in a manner that required Ca^{2+} and membrane depolarization (Ramaswami et al., 1994). Subsequent investigations have confirmed that the same is true for central synapses. Removal of Ca^{2+} in lamprey giant synapses after depletion of synaptic vesicles by high-frequency stimulation resulted in a block of synaptic vesicle recycling and a trapping of the vesicle membrane around the active zones. Addition of Ca^{2+} induced massive endocytosis at periactive zones and complete recovery of the synaptic vesicle clusters, indicating that Ca^{2+} is needed to induce vesicle recycling (Gad et al., 1998).

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Abbreviations: ANTH/ENTH, AP180 N-terminal homology/epsin N-terminal homology; FM1-43, Fei Mao styryl dye 1-43; PIPK5, phosphatidylinositol-4-phosphate-5-kinase; UIM, ubiquitin interacting motif.

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Similarly, dissociation of exo- and endocytosis in murine spinal cord cell cultures by botulinum neurotoxin A did not prevent depolarization-induced retrieval of synaptic vesicle membrane (Neale et al., 1999).

The stimulation of synaptic vesicle endocytosis in central synapses requires an internal Ca^{2+} concentration that is much lower than observed during exocytosis *in vivo*. Vesicular fusion may occur at 10–25 μM Ca^{2+}, but during normal synaptic activity the $[Ca^{2+}]_i$ concentration may exceed 200 μM locally (Neher, 1998; Schneggenburger and Neher, 2005). Endocytosis requires submicromolar concentrations, which are close to or even lower than the intraaxonal Ca^{2+} concentration (Gad et al., 1998; Marks and McMahon, 1998). An increase in intracellular Ca^{2+} concentration accelerates endocytosis in mammalian central synapses (Marks and McMahon, 1998; Wu et al., 2005) see, however, (LoGiudice and Matthews, 2007; von Gersdorff and Matthews, 1994). The Ca^{2+} sensor responsible for triggering endocytosis has been proposed to be calmodulin. This protein stimulates dephosphorylation of several endocytic proteins by calcineurin in nerve terminals (Artalejo et al., 1996; Cousin and Robinson, 2001; Igarashi and Watanabe, 2007). Since recovery of synaptic vesicle pool does not occur in the absence of Ca^{2+}, it is reasonable to suggest that other putative types of endocytosis operating in synapses, such as different forms of bulk endocytosis, are also Ca^{2+}-dependent.

SYNAPTOTAGMIN

The Ca^{2+} sensor for synaptic vesicle fusion is the integral vesicle membrane protein synaptotagmin 1 (Fernandez-Chacon et al., 2001; Koh and Bellen, 2003). It is anchored to the vesicle through N-terminal transmembrane domains and contains two C2 domains, which bind to phospholipids and Ca^{2+} (Bai and Chapman, 2004; Brose et al., 1992). Genetic deletion of synaptotagmin causes severe loss of Ca^{2+}-dependent synchronous release (Geppert et al., 1994). Recent studies have provided new clues for the mechanism for synaptotagmin function in exocytosis (Martens et al., 2007): in response to Ca^{2+} binding, synaptotagmin promotes SNARE-mediated fusion by lowering the activation energy of bilayer–bilayer fusion. This is achieved by inducing a high positive curvature in target membranes following C2-domain membrane insertion (Martens et al., 2007). Synaptotagmin 1 is also believed to act after vesicle fusion as a part of a molecular complex that creates docking sites for clathrin adaptors (Nicholson-Tomishima and Ryan, 2004; Poskanzer et al., 2006). Accordingly, synaptotagmin 1, along with stonin 2, interacts with the μ-subunit of the clathrin adaptor AP2 (Diril et al., 2006; Haucke and De Camilli, 1999; Haucke et al., 2000). Stonin also interacts with scaffolding protein complex Eps15-intersectin, thus facilitating coat formation and dynamin recruitment to the formed clathrin-coated pits (Koh et al., 2007; Martina et al., 2001; Rumpf et al., 2008). Specific residues in the C2B domain of synaptotagmin appear to be important in determining the efficiency of recruitment of endocytic proteins. Thus, mutation of a polylysine motif of the C2B domain alters vesicle size but not endocytic rate whereas mutation of calcium-coordinating aspartate residues alters endocytic rate but not vesicle size (Poskanzer et al., 2006).

Recent imaging experiments have demonstrated that synaptotagmin is present not only on synaptic vesicles, but that is also resides in clusters on the axonal surface. This clustering, however, permits the interchange of synaptotagmin present on the vesicle membrane with the protein available from a large pool on the axonal membrane, at which the concentration of the protein is about 10-fold lower than in synaptic vesicles (Fernandez-Alfonso et al., 2006; Wienisch and Klingauf, 2006; Willig et al., 2006). Binding of clathrin adaptor molecules to synaptotagmin does not occur prior to exocytosis, indicating that exocytosis represents a critical switch in triggering the assembly of clathrin coats. Several lines of evidence discussed below point out that this trigger may be the actual membrane destined for internalization.

LIPID COMPOSITION OF THE MEMBRANE AND ENDOCYTIC PROTEIN RECRUITMENT

Indeed, the composition of lipids and lipid modifications play a critical role in various cellular internalization mechanisms, including synaptic vesicle endocytosis (Di Paolo and De Camilli, 2006; Haucke and Di Paolo, 2007). Clathrin adaptors and many accessory proteins containing (pleckstrin homology) PH and AP180 N-terminal homology/epsin N-terminal homology domains (ANTH/ENTH domains) strongly interact with acidic phospholipids in general, and with $PI(4,5)P_2$ specifically. Proteins containing the ANTH/ENTH domain may function as additional clathrin adaptors and, in addition, control the curvature of the formed clathrin-coated pits (Legendre-Guillemin et al., 2004; Jakobsson et al., in press). In addition, BAR (Bin, amphiphysin, Rvs) domain superfamily proteins, such as endophilin and amphiphysin, which are thought to enhance membrane bilayer bending preferentially, bind to negatively charged lipids and function as membrane associated scaffolds (Gallop et al., 2006; Peter et al., 2004). Other actions of $PI(4,5)P_2$ in endocytosis reflect its effects on the actin cytoskeleton, which is implicated in all internalization pathways (Di Paolo and De Camilli, 2006).

The major cellular mechanism for the generation of $PI(4,5)P_2$ involves the phosphorylation of phosphatidylinositol-4-phosphate PI(4)P at the D-5 position of the inositol ring by the type 1 phosphatidylinositol-4-phosphate-5-kinase (PIPK5). The *PIP5K* gene product consists of three isophormes α, β, and γ (Ishihara et al., 1998). The PIP5K γ isosyme (specifically 661 amino acid splice variant) is highly expressed in the brain and is concentrated in neuronal synapses, where it is used to generate $PI(4,5)P_2$ during synaptic activity (Nakano-Kobayashi et al., 2007; Wenk et al., 2003). It is activated by its interaction with $\beta 2$ and μ subunit of AP2, which bind to the C terminus of the kinase (Krauss et al., 2006; Nakano-Kobayashi et al., 2007). Genetic deletion of *PIP5K* in mice results in decreased levels of $PI(4,5)P_2$ in the brain. An impairment of