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Seong Joo Koo, Dmytro Puchkov & Volker Haucke

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AP180 and CALM

Dedicated endocytic adaptors for the retrieval of synaptobrevin 2 at synapses

Seong Joo Koo,[1,*] Dmytro Puchkov[1] and Volker Haucke[1,2,*]

[1]Institute of Chemistry and Biochemistry; Freie Universität Berlin; [2]Leibniz-Institut für Molekulare Pharmakologie (FMP); Berlin, Germany

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Abbreviations: CALM, clathrin assembly lymphoid myeloid leukemia; AP180, monomeric adaptor protein with 180 kDa; ANTH, AP180 N-terminal homology; SNARE, soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor; VAMP, vesicle-associated membrane protein; SV, synaptic vesicle; AP, action potential; NMR, nuclear magnetic resonance

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*Correspondence to: Seong Joo Koo and Volcker Haucke; Email: seongjookoo@yahoo.com and volker.haucke@fu-berlin.de

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Communication between neurons largely occurs at chemical synapses by conversion of electric to chemical signals. Chemical neurotransmission involves the action potential-driven release of neurotransmitters from synaptic vesicles (SVs) at presynaptic nerve terminals. Fusion of SVs is driven by SNARE complex formation comprising synaptobrevin 2 on the SV membrane and syntaxin 1A and SNAP-25 on the plasma membrane. In order to maintain neurotransmission during repetitive stimulation and to prevent expansion of the presynaptic plasma membrane, exocytic SV fusion needs to be balanced by compensatory retrieval of SV components to regenerate functional vesicles. Our recent work has unraveled a mechanism by which the R-SNARE synaptobrevin 2, the most abundant SV protein and an essential player for exocytic fusion, is recycled from the presynaptic membrane. The SNARE motif of synaptobrevin 2 is directly recognized by the ANTH domains of AP180 and CALM, monomeric endocytic adaptors for clathrin-mediated endocytosis. Given that key residues involved in synaptobrevin 2-ANTH domain complex formation are also essential for SNARE assembly, we propose that disassembly of SNARE complexes is a prerequisite for synaptobrevin 2 retrieval, thereby preventing endocytic mis-sorting of the plasma membrane Q-SNAREs syntaxin 1A and SNAP-25. It is tempting to speculate that perturbed synaptobrevin 2 recycling caused by reduction of CALM or AP180 levels may lead to disease as suggested by the genetic association of ANTH domain proteins with neurodegenerative disorders.

Precise and Rapid Retrieval of Synaptic Vesicle Proteins is Mandatory to Sustain Synaptic Transmission

Upon the arrival of an action potential (AP) at presynaptic terminals, SVs fuse with the plasma membrane at specialized sites termed active zones. Fusion is driven by complex formation between soluble NSF attachment protein receptors (SNAREs) located on the SV and on the plasma membrane, respectively.[1,2] The neuronal SNARE complex consists of the R-SNARE, synaptobrevin 2 (also known as vesicle-associated membrane protein 2, VAMP2) on SVs and the plasma membrane Q-SNAREs, syntaxin 1A and synaptosomal-associated protein (SNAP)-25 (reviewed in ref. 3). Synaptobrevin 2 is the most abundant protein on SVs[3] and is required for activity-induced SV fusion,[4] indicating a crucial role in evoked neurotransmission. Thus, efficient resorting of synaptobrevin 2 after SV fusion is essential to sustain synaptic function. The mechanisms by which synaptobrevin 2 is endocytically resorted has remained elusive. Synaptobrevin 2 lacks typical endocytic sorting signals[5,6] and does not contain potential domains other than its central SNARE helix that could serve as recognition elements[7,8] and, hence, is distinct from other SNARE proteins.

Genetic studies in invertebrate models have suggested a role for AP180 N-terminal homology ANTH domain-containing proteins in synaptobrevin 2 endocytosis.

Caenorhabditis elegans lacking *Unc11*/AP180 no longer accumulates synaptobrevin 2 at synapses[9] and *Drosophila melanogaster* deficient of Like-AP180 (LAP) displays severely impaired SV endocytosis paired with pronounced mis-localization of synaptobrevin 2.[10,11] These studies are consistent with a crucial role for AP180 family members in SV recycling and sorting of synaptobrevin. However, so far it has been difficult to discriminate general endocytic functions of AP180 family members from specific roles in SV protein sorting. Moreover, whether and how AP180 family members physically interact with synaptobrevin has remained unresolved. Lastly, it is unknown whether the presumed role of *Unc11* and LAP in invertebrates is evolutionary conserved in mammals.

Direct Association of Synaptobrevin 2 and AP180/CALM via SNARE Motif Mediates the Recycling of Synaptobrevin 2 at Synapses

We have shown recently that in mammalian central synapses, retrieval of synaptobrevin 2 is regulated by ANTH domain-containing proteins, e.g., the neuron-specific family member AP180 and its ubiquitously expressed homolog clathrin assembly lymphoid myeloid leukemia (CALM).[12] Depletion of AP180 and CALM in hippocampal neurons led to selective accumulation of synaptobrevin 2 on the neuronal surface but did not affect the distribution of other SV proteins such as the vesicular glutamate transporter 1 (vGLUT1). The function of AP180 and CALM appears to be at least partially redundant as neurons lacking both of these factors displayed more severe sorting defects. AP180-depleted neurons also showed enlarged and more heterogeneously sized SVs, an effect that is in good agreement with observations in invertebrate systems[9-11] and similar to the phenotype seen in synaptobrevin 2-null primary neurons.[13] Occasionally, tubular structures could be observed in AP180 deficient nerve terminals (**Fig. 1**). The identity of these structures or the mechanisms by which these are generated are presently unclear. These tubules may represent endosomal or plasma membrane-derived endocytic intermediates, indicating a partial shift to alternative modes of SV regeneration requiring endosomal processing. Whether this is indeed the case will have to await further studies.

We also showed that the retrieval of synaptobrevin 2 by AP180 and CALM involves the direct association with the ANTH domains of both proteins. Convergent evidence from biochemical mapping analysis, direct in vitro binding assays including surface plasmon resonance and peptide SPOT arrays, as well as structural studies by nuclear magnetic resonance (NMR) spectroscopy revealed that AP180- and CALM-ANTH recognize identical determinants within the N-terminal half of the synaptobrevin 2 SNARE helix. NMR-based structural studies indicate that overlapping sites within synaptobrevin 2 are involved in its association with ANTH domain-containing endocytic proteins as well as in SNARE complex formation. When one of these residues, methionine 46, was mutated to alanine binding of synaptobrevin 2 to ANTH domains was drastically reduced. More importantly, M46A mutant synaptobrevin 2 fused to a pH-sensitive green fluorescence protein (GFP) derivative ("pHluorin")[14] failed to be endocytically retrieved following stimulation-induced exocytic membrane insertion. This mechanism likely ensures that only free synaptobrevin 2 devoid of its SNARE binding partners is sorted to recycling SVs.

Implications and Questions

Taken together, our study has unraveled how synaptobrevin 2 is endocytically resorted during exo-endocytic cycling of SV membranes at mammalian central synapses. This novel mechanism involves the direct recognition of the N-terminal half of the SNARE helix within synaptobrevin 2 by the ANTH domains of AP180 and CALM and thus is distinct from previously described mechanisms for the recognition of other SNARE proteins that involve non-SNARE determinants including the Habc domain of vti1b and the longin domain of VAMP7.[7,8] We predict that other brevin-family SNAREs that lack non-SNARE domains may be recognized by similar modes of interaction consistent with the fact that SNARE proteins are found in many different organelles in the cell.

Given that the endocytic sorting signal within synaptobrevin 2 overlaps with the SNARE complex interface only disassembled synaptobrevin 2 can be internalized by AP180 and CALM. Such a mechanism presumably has evolved to prevent missorting of the plasma membrane SNAREs syntaxin and SNAP-25, while ensuring synaptobrevin 2 retrieval. This hypothesis is further supported by previous works showing that syntaxin 1A is excluded from recycling SV membranes and instead is retained on the neuronal plasma membrane.[15] Moreover, a synaptobrevin 2 mutant impaired in SNARE complex disassembly fails to get internalized from the plasma membrane.[16] When and where disassembly of cis-SNARE complexes occurs is an open question. This could conceivably happen either at the active zone, thereby regenerating free syntaxin 1A, which could be reused subsequent rounds of SNARE-driven membrane fusion or at the surrounding peri-active zone where recycling of SV proteins occurs (**Fig. 2**).

Recognition of synaptobrevin 2 by its specific endocytic adaptors, AP180 and CALM provides further support to the hypothesis that SVs lose their identity during exocytic fusion and are recycled piecemeal by cargo-specific mechanisms that involve dedicated sorting adaptors such as stonin 2 [17-19] and endophilin[20] (**Fig. 2**). Our results are also consistent with the view that clathrin-mediated endocytosis is the major retrieval pathway at mammalian central synapses.[21,22] How endocytic SV reformation and sorting of SV components are coordinated is unknown. SV proteins could either be internalized individually or coalesce into preformed clusters that are then retrieved during subsequent rounds of exo-endocytosis. Several lines of evidence suggest that SV proteins form clusters on the plasma membrane. Such clusters have been observed by stimulation emission depletion (STED) microscopy for synaptotagmin 1,[23] synaptophysin and the vesicular GABA transporter (vGAT) [24] and may correspond to the so-called readily retrievable pool of vesicles on the neuronal surface.[25] Post-fusion clustering

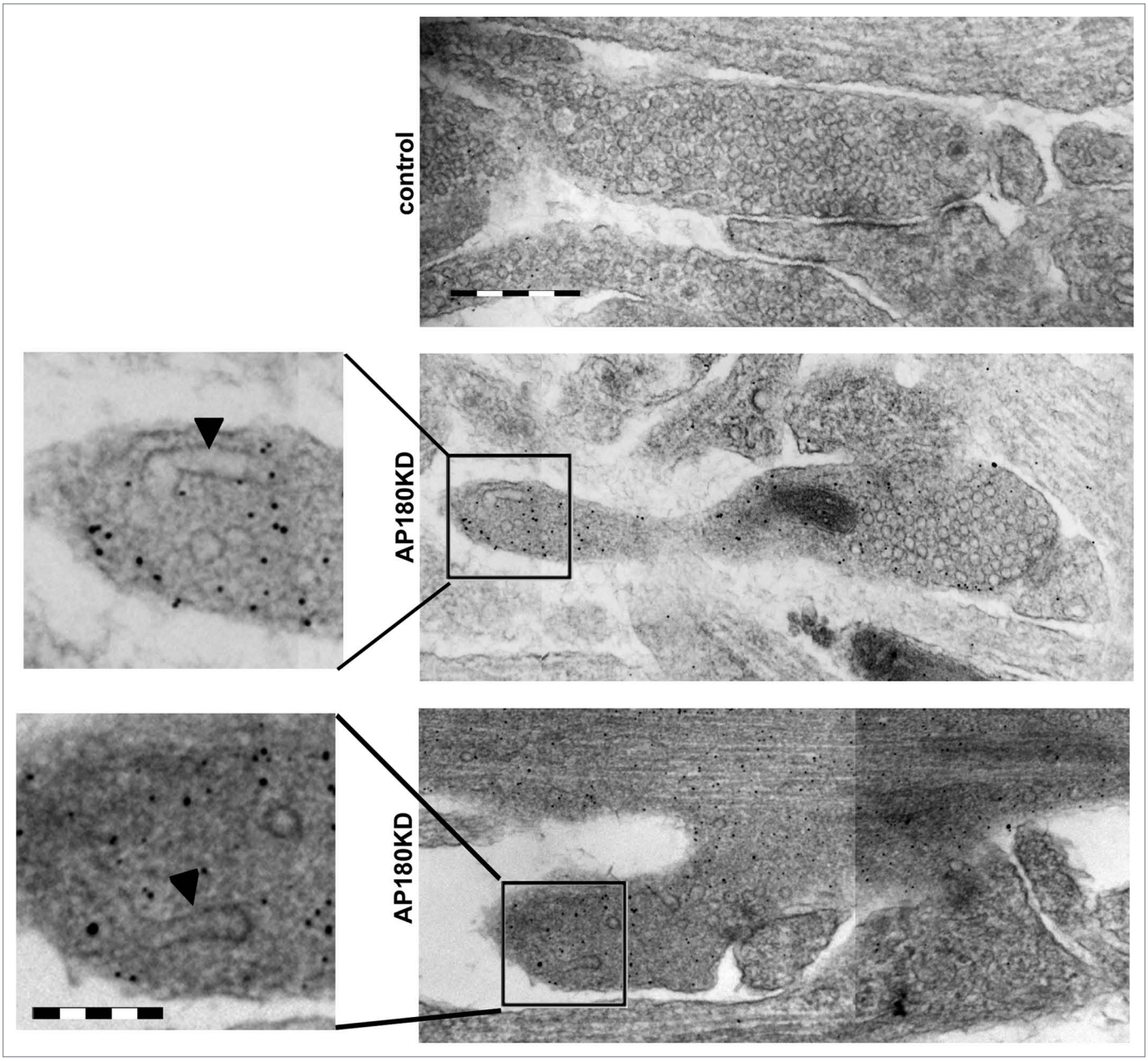

Figure 1. Representative electron micrographs of control and AP180-depleted synapses. Arrowheads in the enlarged insets illustrate tubular structures occasionally found in AP180-depleted nerve terminals. Such structures were not normally seen in synapses from control neurons. Morphology and SV density were unchanged but SVs appeared slightly larger and more heterogeneous in synapses depleted with AP180.[12] Hippocampal neurons were cotransfected with AP180 siRNA and an eGFP-encoding plasmid. Six to eight days after transfection neurons were fixed with 4% paraformaldehyde before permeabilization by freeze-cracking in liquid nitrogen. Samples were labeled with anti-GFP antibody and NANOGOLD® particles, post-fixed with 2% glutaraldehyde, gold-enhanced and processed for electron microscopy. Images were taken using Zeiss 910 electron microscope. Neurites and terminals of control or KD neurons were identified by absence/presence of intense immunogold labeling scattered in the cytoplasm. Scale bar, 500 nm; 200 nm for the inset; KD, knockdown.

of SV proteins is also supported by the formation of detergent-insoluble heteromultimeric SV protein complexes.[26] In agreement with this view, lack of synaptophysin, the second most abundant synaptic vesicle protein and close binding partner of synaptobrevin 2 [27] impairs retrieval of SVs,[28,29] in particularly synaptobrevin 2 endocytosis.[28] Whether or not synaptophysin-dependent retrieval of synaptobrevin 2 operates in parallel or cooperatively with AP180/CALM-based mechanisms remains an important question for future studies.

Is the function of AP180 and CALM restricted to their role as specific endocytic sorting adaptors for synaptobrevin 2 and other brevin SNAREs? Although the answer to this question remains open it is interesting to note that recent systematic genome-wide association studies

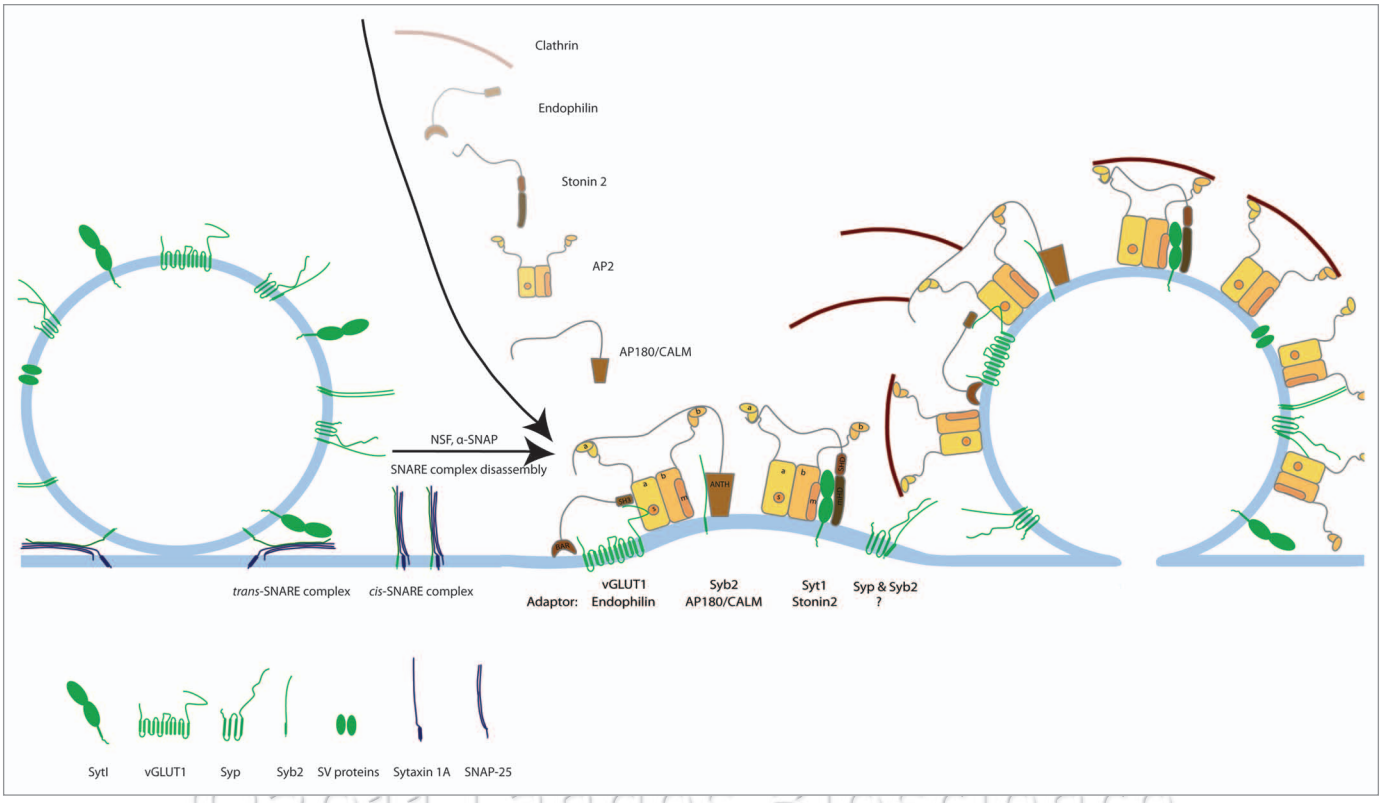

Figure 2. Hypothetical model detailing mechanisms involved in cargo-specific endocytic sorting of SV proteins. Upon the arrival of an action potential at the nerve terminal SVs fuse with the presynaptic membrane via a SNARE-dependent mechanism. As a result cis-SNARE complexes comprising synaptobrevin 2, syntaxin 1A and SNAP-25 remain on the plasma membrane. For new rounds of exocytosis, cis-SNARE complex are disassembled via NSF with its cofactor α-SNAP, releasing free synaptobrevin 2 molecules. Endocytic sorting of SV proteins on the plasma membrane requires dedicated endocytic adaptors: vGLUT1 recognition by AP2 and endophilin,[20] synaptobrevin 2 binding to AP180 and CALM,[12] and synaptotagmin 1 sorting by AP2 and stonin 2.[17-19] AP180 and CALM directly recognize the SNARE motif of synaptobrevin 2, perhaps to ensure that only free rather than SNARE-complexed synaptobrevin 2 is endocytically resorted to while syntaxin 1A and SNAP-25 are left behind. Recycling of synaptobrevin 2 has been shown to require synaptophysin although the molecular details of this mechanism remain unknown.[28] SV cargo becomes concentrated in clathrin-coated pits containing clathrin, endocytic adaptors and accessory proteins resulting in SV reformation. SV, synaptic vesicle; SytI, Synaptotagmin 1; vGLUT1, vesicular glutamate transporter 1; Syp, synaptophysin; Syb2, synaptobrevin 2; NSF, N-ethylmaleimide-sensitive factor; α-SNAP, soluble NSF attachment proteins; mHD, μ homology domain; SHD, stonin homology domain.

have identified CALM as a potential susceptibility associated with increased risk for suffering from Alzheimer disease.[30] Moreover, AP180 protein levels have been shown to be drastically reduced in Alzheimer patients,[31-33] arguing for a potential role of ANTH domain-containing proteins in Alzheimer disease. If and how AP180 and CALM are directly involved in the onset of the disease is unclear. CALM and/or AP180 may regulate amyloid precursor protein (APP) trafficking or Aβ generation.[34] Alternatively, impaired synaptic function could result from mis-sorting of synaptobrevin 2 due to alterations in AP180 or CALM expression, localization or activity, thereby indirectly causing synaptic malfunction and disease. These remain interesting and important questions for future studies.

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