Synaptic Vesicle Endocytosis and Endosomal Recycling in Central Nerve Terminals

Synaptic vesicle endocytosis and endosomal recycling in central nerve terminals: Discrete trafficking routes?

Michael A. Cousin

Address
Centre for Integrative Physiology,
Hugh Robson Building,
George Square,
University of Edinburgh,
Edinburgh,
Scotland, EH8 9XD
Tel: +44131 6503259
Fax: +44131 6506527
Email: M.Cousin@ed.ac.uk

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Abstract

Synaptic vesicle (SV) retrieval from the presynaptic plasma membrane occurs via a variety of different and complementary modes. The dominant retrieval mode during high intensity stimulation is activity-dependent bulk endocytosis (ADBE). ADBE involves the generation of endosomes direct from the plasma membrane which then donate membrane and cargo to form SVs that replenish the reserve SV pool. Recent evidence has suggested that ADBE may involve an additional endosomal processing step to produce a mature, functional SV. This suggests that ADBE may utilise key molecules or indeed whole pathways from classical endocytic recycling routes that are ubiquitous across all cell types. This review will assess the current evidence for a contribution of endocytic recycling to the SV life cycle, with a particular focus on ADBE. In doing so it highlights points where both routes may either converge or exploit existing mechanisms to ensure efficient generation of SVs during high intensity stimulation.

Neurotransmission is triggered by the evoked fusion of synaptic vesicles (SVs) with the presynaptic plasma membrane on action potential stimulation. The efficient retrieval of SVs after their fusion is equally essential for the maintenance of neurotransmission, since in typical small central nerve terminals they are highly limited in number. Several SV retrieval modes exist in nerve terminals, which are differentially triggered by a range of physiological stimuli. For example, single action potentials can trigger a form of SV retrieval with subsecond kinetics (ultrafast endocytosis) that occurs directly adjacent to the site of SV fusion (Watanabe and others 2013a; Watanabe and others 2013b), whereas slightly stronger stimulation favours the slower clathrin-mediated endocytosis (CME) mode of retrieval (Granseth and others 2006). However when stimulation intensities increase to a stage that these SV retrieval modes are saturated, an additional pathway is triggered, called activity-dependent bulk endocytosis (ADBE, Clayton and Cousin 2009a). ADBE retrieves large regions of the plasma membrane at regions distal to SV fusion sites. These invaginations are formed into endosomes from which SVs can be subsequently generated (Figure 1). Bulk endosome-derived SVs are fully functional and once formed enter the recycling pool of SVs. The recycling pool is defined as SVs that are mobilised by action potential stimulation and is comprised of both the readily releasable pool (RRP) and the reserve pool (Rizzoli and Betz 2005; Royle and Lagnado 2003). SVs derived from bulk endosomes exclusively replenish the reserve pool (Cheung and others 2010) which is mobilised by the same stimulation intensities required to trigger ADBE. Thus in simplest terms ADBE can be viewed as a mechanism that replenishes the reserve SV pool after its depletion by high intensity stimulation. This is an important process, since ADBE is the dominant mode of SV retrieval during intense stimulation (Clayton and others 2008). This means it should play a key role in neuronal events dependent on such stimuli, including physiological mechanisms such as changes in synaptic strength via long-term potentiation and also in pathophysiological events such as epileptic seizure.

Molecular mechanism of SV generation by ADBE

The generation of SVs via ADBE occurs via at least two discrete stages. Firstly there is the direct formation of the bulk endosome from the plasma membrane and the subsequent generation of SVs from these endosomes (Figure 2). The molecular mechanism of ADBE is only now starting to be elucidated, with a number of key molecules identified over the past 5 years that are essential for both bulk endosome and SV generation respectively (Clayton and Cousin 2009a).

Bulk endosome formation is triggered by intense neuronal activity. This increase in neuronal activity correlates with activation of the calcium-dependent protein phosphatase calcineurin (Clayton and others 2009). Multiple studies have suggested calcineurin activity is essential for ADBE (Clayton and others 2009; Xue and others 2012). Calcineurin has numerous substrates within central nerve terminals (Cousin and Robinson 2001), however the key substrate for ADBE appears to be the large GTPase dynamin I. This is because dynamin I dephosphorylation only occurs during intense neuronal

activity (thus correlating with triggering of ADBE), and mutagenesis of the two key phosphorylation sites on dynamin I dephosphorylated by calcineurin arrest this process (Clayton and others 2009). Activity-dependent dynamin I dephosphorylation triggers an interaction with the F-BAR (bin-amphiphysin-RVS) protein syndapin (Anggono and others 2006). Syndapin recruitment is essential for ADBE, since manoeuvres that either inhibit its dephosphorylation-dependent interaction with dynamin I, or ablate syndapin expression result in arrest of the process (Clayton and others 2009). After stimulation the subsequent sequential rephosphorylation of dynamin I by cyclin-dependent kinase 5 and then by glycogen synthase kinase 3 is also essential for multiple cycles of ADBE to occur (Clayton and others 2010). Thus the phosphorylation status of dynamin I is critical for both the triggering (calcineurin-mediated dephosphorylation) and maintenance (cyclin-dependent kinase 5 / glycogen synthase kinase 3) of ADBE.

As stated above, dynamin I is also a GTPase and this enzymatic activity is essential for SV fission from the plasma membrane during both CME and ultrafast endocytosis in central nerve terminals (Newton and others 2006; Ferguson and others 2007; Watanabe and others 2013b). Dynamin GTPase activity was also proposed to be essental for the fission of nascent bulk endosomes during ADBE (Clayton and others 2009; Xue and others 2012). However nerve terminals from either dynamin I knockout or dynamin I / III double knockout mice (Hayashi and others 2008; Raimondi et al 2011) or containing actutely inactivated dynamin in *Drosophila* (Kasprowicz and others 2014) could still generate intracellular cisternal structures similar in appearance to bulk endosomes. Furthermore recent studies have shown that pharmacolgical dynamin inhibtors (which were used to determine the role of dynamin GTPase activity in ADBE, Clayton and others 2009; Xue and others 2012) may inhibit endocytosis via a dynamin-independent mechanism (Park and others 2013). Therefore the role of dynamin GTPase activity in bulk endosome generation during ADBE is still undetermined.

The generation of bulk endosomes from the nerve terminal plasma membrane occurs independently from the action of the classical CME machinery, such as clathrin and the adaptor protein complex AP-2 (Heerssen and others 2008; Kasprowicz and others 2008; Kononenko and others 2014). However clathrin is essential for the generation of SVs from bulk endosomes, since its acute inactivation arrests this process in *Drosophila* (Heerssen and others 2008; Kasprowicz and others 2008). Interestingly, recent studies have suggested that the plasma membrane adaptor complex AP-2 may be required for SV generation from bulk endosome-like intermediates in central nerve terminals (Kononenko and others 2014). This suggests that sorting of SV cargo occurs at the level of the bulk endosome and not the plasma membrane (Box 1). This is supported by previous studies that demonstrated an essential requirement for adaptor protein complexes in SV generation from bulk endosomes. These studies were facilitated by novel assays that specifically tracked the generation and fate of bulk endosome-derived SVs (Cheung and others 2010, Figure 3). Using these assays it was discovered that there was a dual requirement for the adaptor protein complexes, AP-1 and AP-3 (Cheung and Cousin 2012).

These adaptor protein complexes usually mediate the sorting of cargo from different intracellular compartments, such as endosomes. Inhibition of their recruitment by brefeldin A ablated SV generation (Figure 3) and when expression of key subunits from these complexes was individually knocked down using shRNA, they produced identical phenotypes. Importantly simultaneous knockdown of these subunits did not produce an additional reduction in reserve pool replenishment, meaning both AP-1 and AP-3 are part of the same SV generation pathway in ADBE. Thus the clustering and sorting of SV cargo during ADBE occurs at the level of the bulk endosome and not at the plasma membrane. This also suggests that plasma membrane cargo may be inadvertently internalised during ADBE and will have to be either returned to the cell surface at a later stage or alternatively destroyed via a degradative pathway.

Other key molecular events have been identified that are required for SV generation from the bulk endosome. For example, SV generation is dependent on the fluid phase uptake of extracellular calcium during ADBE (Cheung and Cousin 2013). This was shown by buffering nerve terminal calcium after bulk endosome formation and confirmed by chelating accumulated calcium that was inside newly formed bulk endosomes. This calcium is released from bulk endosomes on their acidification, which triggers activation of calcineurin. Multiple calcineurin substrates exist in central nerve terminals (Cousin and Robinson 2001) however at this stage it is unclear which dephosphorylation event is critical for budding to occur. Thus a number of key molecular events have begun to be identified that are required for SV generation during ADBE (Figure 2).

Endocytic recycling

The molecular mechanism of ADBE hints at similarities with classical endocytic recycling routes. For example, the dual requirement for endosomal adaptor protein complexes suggests that SV cargo may shuttle via an additional sorting compartment, since two independent budding events may be required. Furthermore the fact that both endocytic organelles and bulk endosomes expel calcium on acidification (Gerasimenko and others 1998) suggests ADBE may either exploit the existing endosomal recycling machinery or utilise similar structures.

Classical endocytic recycling serves a number of essential cellular purposes. For example it is required for the uptake of nutrients bound to plasma membrane receptors, their separation, the processing of internalised cargo and the return of receptors from the cell surface. It is also essential for trafficking molecules for degradation, for maintaining membrane homeostasis and also for controlling the type and number of receptors and transport proteins on the cell surface (Box 1).

The first step in endocytic recycling usually involves the fusion of newly formed endocytic vesicles with an early endosome (also known as a sorting endosome). This fusion event is dependent on a number of molecular factors including the small GTPase Rab5, early endosome antigen 1 and

phosphatidylinositol-3-phosphate, generated by the lipid kinase phosphatidylinositol-3-kinase (Grant and Donaldson 2009). The mild acidity of this compartment allows the release of bound ligands from their receptors (Maxfield and McGraw 2004). After this point cargo can be trafficked via two different routes, either a "fast" or "slow" recycling pathway. The fast recycling route traffics cargo directly back to the plasma membrane from the sorting endosome. Alternatively cargo can be trafficked via a slower route which involves movement through the endocytic recycling compartment (ERC) and then via the recycling endosome before return to the cell surface (Li and DiFiglia 2012) (Figure 4).

One of the key elements of fast recycling is the requirement for the small GTPase Rab4. Knockdown studies or experiments where dominant negative Rab4 mutants were expressed have resulted in reduced cargo traffic back to the membrane (Deneka and others 2003; Yudowski and others 2009; Cheng and others 2013). In addition to Rab4, Rab35 also regulates this rapid recycling route (Grant and Donaldson 2009). Rab35 is recruited to coated pits by connecdenn, a scaffolding protein that shares interactions with the adaptor complex AP-2 and src-homology 3 domain containing endocytosis proteins (Allaire and others 2006). Connecdenn is also a GTP exchange factor (GEF) for Rab35 and therefore facilitates its function (Allaire and others 2010).

As stated above, the slow cargo recycling route involves traffic from the ERC back to the plasma membrane. In this route, early endosome maturation results in the extension of tubules which become the ERC, whereas the remaining early endosome eventually becomes the multivesicular body (MVB) (Grant and Donaldson 2009). The ERC is defined by the presence of the GTPase Rab11 and traffic from Rab5-positive early endosomes to Rab11-positive ERCs is thought to be mediated by a family of ATPases called eps15 homology domain containing proteins (EHDs) (Naslavsky and Caplan 2011). Another Rab family member, Rab22a, is also proposed to be required for cargo traffic from the early endosome to the ERC (Magadan and others 2006).

Traffic from the ERC back to the plasma membrane is complex and may involve multiple separate routes. For example an Arf6-dependent route exists, where tubular endosomes extend out from the ERC to mediate traffic back to the plasma membrane (Grant and Donaldson 2009). Other routes back to the plasma membrane involve carrier vesicles formed directly from the ERC, the fission of which may be controlled by EHD1. EHD1 is proposed to act in a similar manner to dynamin, namely it utilises energy from ATP hydrolysis to provide mechanical force for vesicle fission (Daumke and others 2007). It also interacts with BAR domain-containing proteins such as syndapin and amphiphysin, whose functions involve actin organisation and membrane deformation (Braun and others 2005; Pant and others 2009). Thus EHD1 has been proposed to be a "gatekeeper" for the ERC for plasma membrane recycling of cargo (Naslavsky and Caplan 2011).

Do classical endocytic recycling routes contribute towards SV recycling?

Endocytic recycling occurs in all cell types, however the contribution of this mechanism to SV turnover in central nerve terminals is still debated. The first published molecular inventory of a SV in a typical small central nerve terminal contained a large number of proteins that were immediately recognised as classical endosomal recycling molecules (Takamori and others 2006). In a subsequent study which investigated the Rab content on a population of highly pure SVs, again a considerable number of endocytic Rabs were identified, including Rab4, Rab5, Rab7, Rab10, Rab11b, and Rab14 (Pavlos and others 2010). Furthermore when this subset of Rab proteins were expressed in primary neuronal culture, almost all localised to nerve terminals. However it was intriguing that this was only a partial co-localisation, with most Rabs (Rab4b, Rab5a, Rab10, Rab11b, Rab14) present only in a subset of nerve terminals, suggesting that endosomal recycling may potentially only occur in certain nerve terminals.

Of all the endocytic Rabs, Rab5 is the most investigated in terms of SV recycling. Both endogenous and exogenous Rab5 co-localise with SV proteins in mature hippocampal cultures (Rizzoli and others 2006; Star and others 2005) and Rab5 was also found in purified populations of central nerve terminals (de Hoop and others 1994). Rab5 is retained within nerve terminals during stimulation of primary neuronal cultures, whereas the SV Rab, Rab3, transiently trafficked into the axon (Star and others 2005). This suggests that Rab5 was anchored to an intracellular compartment such as an endosome and was not present in large amounts on SVs. When the function of Rab5 was manipulated however, effects on SV recycling have been observed. For example, nerve terminals at a *Drosophila* neuromuscular synapse that expressed a dominant negative form of Rab5 showed disrupted uptake and release of the dye FM1-43 at low frequency stimulation and a decrease in the SV recycling pool during high frequency stimulation (Wucherpfennig and others 2003). In the same study overexpression of wild-type Rab5 increased SV turnover, suggesting trafficking via a Rab5 compartment facilitated SV turnover. However when a similar experiment was performed in cultured mammalian neurons either an opposite outcome was observed (Star and others 2005) or there was no effect (Shin and others 2008). Nevertheless, a non GTP binding mutant of Rab5 did affect the speed of SV cargo retrieval (Shin and others 2008). Finally expression of constitutively active Rab5 mutant has no effect on neurotransmitter release in the nematode *C. elegans* (Sasidharan and others 2012). Therefore information on the role for Rab5 in SV recycling is sparse and difficult to interpret due to differences in the mutants employed and the systems investigated.

More recent work has suggested that classical early endosomes may contribute specifically towards RRP replenishment. In this study a fragment of syntaxin 13 was used to interfere with the fusion of endocytic vesicles with endosomes which resulted in a greatly reduced size of the RRP (Hoopmann and others 2010). More direct evidence of a role for sorting endosomes in SV recycling came from studies in *Drosophila* investigating the Rab35 GAP *skywalker* (Uytterhoeven and others 2011).

Skywalker mutants displayed a large increase in nerve terminal endosome number on strong stimulation. This increase was not due to increased ADBE, since no additional dextran uptake was observed. Interestingly constitutively active Rab5, 23 and 35 all phenocopied *skywalker* defects, with FM1-43 accumulated in nerve terminal substructures and the dye was released on subsequent stimulation. A model was proposed where Rab5, Rab23 and Rab35 are required for the efficient entry and exit of SV cargo via a sorting endosome that is distinct from a classical bulk endosome. The *skywalker* gene product was proposed to restrict traffic via this route by activating Rab35 GTPase activity, thus these mutants display enhanced neurotransmitter release due to more endosomal sorting. In agreement knockdown of the Rab35 GEF connecdenn in neurons greatly reduced KCl-evoked FM4-64 dye uptake, suggesting that modulation of cargo flow through sorting endosomes impacts on SV recycling (Allaire and others 2006). Thus in this model increased endosomal recycling of SV cargo facilitates SV turnover, as opposed to ADBE, where traffic via bulk endosomes results in a transient sequestration of cargo from the recycling machinery.

The question as to whether central nerve terminals utilise either the rapid or slow route of recycling cargo back to the plasma membrane during SV turnover is unclear due to the lack of available studies. For conventional SV recycling it would seem unlikely, since there is already a well characterised route for the traffic and fusion of SVs subsequent to their formation. Nevertheless Rab4 is present on SVs (Pavlos and others 2010) and it may control the formation of synaptic-like microvesicles (which are similar to small SVs) from sorting endosomes in PC12 cells in conjunction with Rab10 (de Wit and others 2001). The Rab11-dependent slow recycling pathway is not thought to extend into axonal nerve terminals (Li and DiFiglia 2012) however a potential role for Rab11 in nerve terminal function has been shown in *Drosophila*. In this study overexpression of Rab11 rescues neurotransmission defects that occur on expression of a mutant form of huntingtin (Steinert and others 2012). It is too early to speculate on whether the molecular mechanism of rescue is due to direct effects on SV recycling, however the role of Rab11 in nerve terminal function is a topic which requires further investigation.

ADBE and endosomal recycling routes

ADBE generates endosomes directly from the plasma membrane, thus it may benefit from utilisation of the existing machinery provided by the classical endosomal recycling pathway. However what evidence is there that ADBE either utilises or exploits the classical endosomal recycling machinery? This first line of evidence is that ADBE only occurs in a subset of central nerve terminals (Clayton and Cousin 2009b; Wenzel and others 2012). The proportion of nerve terminals that undergo ADBE correlate well with the number that also contain sorting endosome markers (Pavlos and others 2010), suggesting that the presence of the classical endosomal recycling machinery may be a prerequisite for ADBE to occur. In support Rab5 is localised to cisternal, ADBE-like, structures in Drosophila nerve

terminals (Wucherpfennig and others 2003). Furthermore an exogenously expressed early endosomal marker (EGFP-FYVE) disappeared completely on SV depletion during a temperature-sensitive endocytoic block in *shibire*, suggesting these endosomes may be transient and dependent on ADBE (Wucherpfennig and others 2003). However as previously discussed, other studies have hinted that the appearance of nerve terminal early endosomes in *Drosophila* can be activity-dependent, but independent of ADBE (Uytterhoeven and others 2011).

In simplest terms, to exploit the endosomal recycling system bulk endosomes (or carriers generated from them) would have to fuse with classical sorting endosomes. The strongest current evidence that this may occur comes from studies showing that both AP-1 and AP-3 are required for SV generation during ADBE (Cheung and Cousin 2012). This suggests that an endosomal intermediate sorting step may be required for either SV maturation or cargo processing during ADBE. This is supported by evidence showing that bulk endosomes can fuse with either each other or early endosomes. In these studies bulk endosomes isolated from central nerve terminals underwent apparent fusion *in vitro* in an ATP-dependent manner (Rizzoli and others 2006). This suggests that bulk endosomes have the ability to undergo homotypic fusion to generate endosomal compartments. This has yet to be demonstrated *in vivo*, where the number of bulk endosomes labelled with the fluid phase marker horse radish peroxidase (HRP) did not change over a 30 minute time period in nerve terminals of cultured neurons (Cheung and others 2010).

One intriguing aspect of homotypic endosome fusion is that is appears to be dependent on calcium. Furthermore localised calcium increases seem to be essential for fusion to occur, since fast binding calcium buffers such as BAPTA can inhibit this process, but not slower buffers such as EGTA (Holroyd and others 1999). Interestingly FM dye-labelled bulk endosomes extracted from central nerve terminals could only fuse with endosomes from the PC12 neuroendocrine cell line when slow calcium buffers were present, but not fast (Rizzoli and others 2006). This has close parallels to SV generation via ADBE, where a leak of accumulated calcium from acidifying organelles is essential for the event to occur (Cheung and Cousin 2013). It is therefore conceivable that calcium-dependent SV generation during ADBE may not be due to SV budding from bulk endosomes, but rather the fusion of endocytic carriers or indeed the bulk endosome itself with a sorting endosome (Figure 5).

An alternative possibility for bulk endosomes to enter the endocytic recycling system is for them to acquire molecular markers to become sorting endosomes. No evidence is currently available regarding whether or not this occurs, however phosphatidylinositol 3-kinase activity is required for SV generation via ADBE at the amphibian neuromuscular junction (Richards and others 2004). Thus the localised production of phospholipids on bulk endosomes could be a mechanism which permits the recruitment of sorting endosome molecules to allow their entry into the endocytic recycling system.

Recent studies examining SV reformation from bulk endosome-like structures in central nerve terminals indicated a potential role for the plasma membrane adaptor protein complex AP-2 (Kononenko and others 2014). This suggested that a large amount of plasma membrane cargo may have been inadvertently accumulated during bulk endosome generation. Since this cargo will not be packaged into SVs by the clathrin/adaptor complex machinery, it either has to be degraded or returned to the cell surface. Both of these alternatives should only occur after the bulk endosome has donated all SV cargo to newly formed vesicles. Potential routes for degradation include trafficking of spent bulk endosomes via the lysosomal pathway, however no evidence is currently available to suggest that bulk endosomes are degraded. Nevertheless it could be envisaged that classical sorting endosomes mature into late endosomes and enter the degradative pathway after their initial fusion with bulk endosomes (Grant and Donaldson 2009).

As stated above, plasma membrane cargo that was inadvertently taken up during ADBE may be returned to the cell surface via the endocytic recycling machinery, for example via the slow Rab11 pathway. Constitutive recycling of bulk endosomes to the cell surface is unlikely however, since they are present in high numbers 30 minutes after their formation by stimulation (Cheung and others 2010). However it has been suggested that bulk endosomes are recycled in an activity-dependent manner. In both lap (equivalent to AP180 in mammals) and intersectin mutants in *Drosophila* there is an inhibition of overall endocytosis events with a heterogeneity in the size of vesicles produced (Koh and others 2004; Zhang and others 1998). In both cases large miniature EPSC events were observed, suggesting fusion of larger vesicles with the plasma membrane. However there was no evidence presented to prove that these larger vesicles were *bone fide* bulk endosomes and not simply malformed vesicles produced by dysfunctional CME. In the Calyx of Held, which is a large atypical central nerve terminal, large single event capacitance increases were observed on stimulation, which were proposed to be due to the fusion of large vesicles generated by prior compound SV fusion (He and others 2009). However these structures are not by definition bulk endosomes either. Indeed ADBE was proposed to occur in response to these compound fusion events. Thus no evidence currently exists to suggest that bulk endosomes fuse with the plasma membrane. In agreement, no depletion of bulk endosomes labelled with horse radish peroxidise was observed during multiple rounds of high intensity stimulation in primary neuronal culture (Cheung and Cousin 2013). Furthermore no activity-dependent disappearance of dextran-positive puncta has been observed in multiple studies (Clayton and Cousin 2009b; Wenzel and others 2012). However a possibility still exists that that plasma membrane cargo is being recycled to the cell surface via a Rab11-dependent carriers and that these fluid phase markers are retained inside sorting endosomes (Figure 5). This question will not fully be resolved until firstly plasma membrane cargo are unequivocally shown to be accumulated during ADBE and secondly, when genetic reporters are designed to track their traffic in real time after formation of bulk endosomes.

Conclusions

SV recycling is a highly specialised process, exquisitely coupled to neuronal activity. Because of this questions regarding the requirement, if any, for the ubiquitous endocytic recycling system have been raised. Of all the SV recycling modes ADBE would benefit most from this system, since from first principles the duplication of similar trafficking compartments would not seem energetically favourable. However for this link to be proven (or disproven) key questions need to be addressed. These include, do ADBE and endosome recycling occur in the same nerve terminals? Does interference with key endosome recycling molecules perturb ADBE and vice versa? Finally is plasma membrane cargo accumulated by ADBE and if so how is it returned to the cell surface? Once these questions have been addressed a clearer picture should emerge regarding whether ADBE and endocytic recycling form a working partnership or remain mutually exclusive.

References

Allaire PD, Marat AL, Dall'Armi C, Di Paolo G, McPherson PS, and Ritter B. 2010. The Connecdenn DENN domain: a GEF for Rab35 mediating cargo-specific exit from early endosomes. Mol Cell 37:370-82.

Allaire PD, Ritter B, Thomas S, Burman JL, Denisov AY, Legendre-Guillemin V, Harper SQ, Davidson BL, Gehring K, and McPherson PS. 2006. Connecdenn, a novel DENN domain-containing protein of neuronal clathrin-coated vesicles functioning in synaptic vesicle endocytosis. J Neurosci 26 :13202-12.

Anggono V, Smillie KJ, Graham ME, Valova VA, Cousin MA, and Robinson PJ. 2006. Syndapin I is the phosphorylation-regulated dynamin I partner in synaptic vesicle endocytosis. Nat Neurosci 9 :752-60.

Braun A, Pinyol R, Dahlhaus R, Koch D, Fonarev P, Grant BD, Kessels MM, and Qualmann B. 2005. EHD proteins associate with syndapin I and II and such interactions play a crucial role in endosomal recycling. Mol Biol Cell 16 :3642-58.

Cheng J, Liu W, Duffney LJ, and Yan Z. 2013. SNARE proteins are essential in the potentiation of NMDA receptors by group II metabotropic glutamate receptors. J Physiol 591 :3935-47.

Cheung G and Cousin MA. 2012. Adaptor protein complexes 1 and 3 are essential for generation of synaptic vesicles from activity-dependent bulk endosomes. J Neurosci 32 :6014-23.

Cheung G, and Cousin MA. 2013. Synaptic vesicle generation from activity-dependent bulk endosomes requires calcium and calcineurin. J Neurosci 33 :3370-9.

Cheung G, Jupp OJ, and Cousin MA. 2010. Activity-dependent bulk endocytosis and clathrin-dependent endocytosis replenish specific synaptic vesicle pools in central nerve terminals. J Neurosci 30 :8151-61.

Clayton EL, Anggono V, Smillie KJ, Chau N, Robinson PJ, and Cousin MA. 2009. The phospho-dependent dynamin-syndapin interaction triggers activity-dependent bulk endocytosis of synaptic vesicles. J Neurosci 29 :7706-17.
Clayton EL, and Cousin MA. 2009a. The molecular physiology of activity-dependent bulk endocytosis of synaptic vesicles. J Neurochem 111 :901-14.
Clayton EL, and Cousin MA. 2009b. Quantitative monitoring of activity-dependent bulk endocytosis of synaptic vesicle membrane by fluorescent dextran imaging. J Neurosci Meth 185 :76-81.
Clayton EL, Evans GJ, and Cousin MA. 2008. Bulk synaptic vesicle endocytosis is rapidly triggered during strong stimulation. J Neurosci 28 :6627-32.
Clayton EL, Sue N, Smillie KJ, O'Leary T, Bache N, Cheung G, and others. 2010. Dynamin I phosphorylation by GSK3 controls activity-dependent bulk endocytosis of synaptic vesicles. Nat Neurosci 13 :845-51.
Cousin MA, and Robinson PJ. 2001. The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. Trends Neurosci 24 :659-65.
Daumke O, Lundmark R, Vallis Y, Martens S, Butler PJ, and McMahon HT. 2007. Architectural and mechanistic insights into an EHD ATPase involved in membrane remodelling. Nature 449 :923-7.
de Hoop MJ, Huber LA, Stenmark H, Williamson E, Zerial M, Parton RG, and Dotti CG. 1994. The involvement of the small GTP-binding protein Rab5a in neuronal endocytosis. Neuron 13 :11-22.
de Wit H, Lichtenstein Y, Kelly RB, Geuze HJ, Klumperman J, and van der Sluijs P. 2001. Rab4 regulates formation of synaptic-like microvesicles from early endosomes in PC12 cells. Mol Biol Cell 12 :3703-15.
Deneka M, Neeft M, Popa I, van Oort M, Sprong H, Oorschot V, Klumperman J, Schu, P, and van der Sluijs P. 2003. Rabaptin-5alpha/rabaptin-4 serves as a linker between rab4 and gamma(1)-adaptin in membrane recycling from endosomes. EMBO J 22 :2645-57.
Ferguson SM, Brasnjo G, Hayashi M, Wölfel M, Collesi C, Giovedi S, and others. 2007. A selective activity-dependent requirement for dynamin 1 in synaptic vesicle endocytosis. Science 316:570-4.
Gerasimenko JV, Tepikin AV, Petersen OH, and Gerasimenko OV. 1998. Calcium uptake via endocytosis with rapid release from acidifying endosomes. Curr Biol 8 :1335-8.
Granseth B, Odermatt B, Royle SJ, and Lagnado L. 2006. Clathrin-mediated endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses. Neuron 51 :773-86.
Grant BD, and Donaldson JG. 2009. Pathways and mechanisms of endocytic recycling. Nat Rev Mol Cell Biol 10 :597-608.
Hayashi M, Raimondi A, O'Toole E, Paradise S, Collesi C, Cremona O, and others. 2008. Cell- and stimulus-dependent heterogeneity of synaptic vesicle endocytic recycling mechanisms revealed by studies of dynamin 1-null neurons. Proc Natl Acad Sci 105:2175-80.
He L, Xue L, Xu J, McNeil BD, Bai L, Melicoff E, Adachi R, and Wu LG. 2009. Compound vesicle fusion increases quantal size and potentiates synaptic transmission. Nature 459 :93-7.

Heerssen H, Fetter RD, Davis GW. 2008. Clathrin dependence of synaptic-vesicle formation at the Drosophila neuromuscular junction. Curr Biol 18:401-9.
Holroyd C, Kistner U, Annaert W, and Jahn R. 1999. Fusion of endosomes involved in synaptic vesicle recycling. Mol Biol Cell 10 :3035-44.
Hoopmann P, Punge A, Barysch SV, Westphal V, Buckers J, Opazo F, Bethani I, Lauterbach MA, Hell SW, and Rizzoli SO. 2010. Endosomal sorting of readily releasable synaptic vesicles. Proc Natl Acad Sci 107 :19055-60.
Kasprowicz J, Kuenen S, Miskiewicz K, Habets RL, Smitz L, Verstreken P. 2008. Inactivation of clathrin heavy chain inhibits synaptic recycling but allows bulk membrane uptake. J Cell Biol 182:1007-16.
Kasprowicz J, Kuenen S, Swerts J, Miskiewicz K, Verstreken P. 2014. Dynamin photoinactivation blocks clathrin and α-adaptin recruitment and induces bulk membrane retrieval. J Cell Biol 204:1141-56.
Koh TW, Verstreken P, and Bellen HJ. 2004. Dap160/intersectin acts as a stabilizing scaffold required for synaptic development and vesicle endocytosis. Neuron 43 :193-205.
Kononenko NL, Puchkov D, Classen GA, Walter AM, Pechstein A, Sawade L, and others. 2014. Clathrin/AP-2 mediate synaptic vesicle reformation from endosome-like vacuoles but are not essential for membrane retrieval at central synapses. Neuron 82:981-8.
Li X, and DiFiglia M. 2012. The recycling endosome and its role in neurological disorders. Prog Neurobiol 97 :127-41.
Magadan JG, Barbieri MA, Mesa R, Stahl PD, and Mayorga LS. 2006. Rab22a regulates the sorting of transferrin to recycling endosomes. Mol Cell Biol 26 :2595-614.
Maxfield FR, and McGraw TE. 2004. Endocytic recycling. Nat Rev Mol Cell Biol 5 :121-32.
Naslavsky N, and Caplan S. 2011. EHD proteins: key conductors of endocytic transport. Trends Cell Biol 21 :122-31.
Newton AJ, Kirchhausen T, and Murthy VN. 2006. Inhibtion of dynamin completely blocks compensatory synaptic vesicle endocytosis. Proc Natl Acad Sci 103 :17955-60.
Pant S, Sharma M, Patel K, Caplan S, Carr CM, and Grant BD. 2009. AMPH-1/Amphiphysin/Bin1 functions with RME-1/Ehd1 in endocytic recycling. Nat Cell Biol 11 :1399-410.
Park RJ, Shen H, Liu L, Liu X, Ferguson SM, De Camilli P. 2013. Dynamin triple knockout cells reveal off target effects of commonly used dynamin inhibitors. J Cell Sci 126:5305-12.
Pavlos NJ, Gronborg M, Riedel D, Chua JJ, Boyken J, Kloepper TH, Urlaub H, Rizzoli SO, and Jahn R. 2010. Quantitative analysis of synaptic vesicle Rabs uncovers distinct yet overlapping roles for Rab3a and Rab27b in Ca2+-triggered exocytosis. J Neurosci 30 :13441-53.
Raimondi A, Ferguson SM, Lou X, Armbruster M, Paradise S, Giovedi S, and others. 2011. Overlapping role of dynamin isoforms in synaptic vesicle endocytosis. Neuron 70:1100-14.

Richards DA, Rizzoli SO, and Betz WJ. 2004. Effects of wortmannin and latrunculin A on slow endocytosis at the frog neuromuscular junction. J Physiol 557 :77-91.
Rizzoli SO, Bethani I, Zwilling D, Wenzel D, Siddiqui TJ, Brandhorst D, and Jahn R. 2006. Evidence for early endosome-like fusion of recently endocytosed synaptic vesicles. Traffic 7 :1163-76.
Rizzoli SO, and Betz WJ. 2005. Synaptic vesicle pools. Nat Rev Neurosci 6 :57-69.
Royle SJ, and Lagnado L. 2003. Endocytosis at the synaptic terminal. J Physiol 553 :345-55.
Sasidharan N, Sumakovic M, Hannemann M, Hegermann J, Liewald JF, Olendrowitz C, and others. 2012. RAB-5 and RAB-10 cooperate to regulate neuropeptide release in Caenorhabditis elegans. Proc Natl Acad Sci 109 :18944-9.
Shin N, Jeong H, Kwon J, Heo HY, Kwon JJ, Yun HJ, and others. 2008. LRRK2 regulates synaptic vesicle endocytosis. Exp Cell Res 314 :2055-65.
Star EN, Newton AJ, and Murthy VN. 2005. Real-time imaging of Rab3a and Rab5a reveals differential roles in presynaptic function. J Physiol 569 :103-17.
Steinert JR, Campesan S, Richards P, Kyriacou CP, Forsythe ID, and Giorgini F. 2012. Rab11 rescues synaptic dysfunction and behavioural deficits in a Drosophila model of Huntington's disease. Hum Mol Gen 21 :2912-22.
Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, and others. 2006. Molecular anatomy of a trafficking organelle. Cell 127 :831-46.
Uytterhoeven V, Kuenen S, Kasprowicz J, Miskiewicz K, and Verstreken P 2011. Loss of skywalker reveals synaptic endosomes as sorting stations for synaptic vesicle proteins. Cell 145 :117-32.
Watanabe S, Liu Q, Davis MW, Hollopeter G, Thomas N, Jorgensen NB, and Jorgensen EM. 2013a. Ultrafast endocytosis at Caenorhabditis elegans neuromuscular junctions. eLife 2 :e00723.
Watanabe S, Rost BR, Camacho-Perez M, Davis MW, Sohl-Kielczynski B, Rosenmund C, and Jorgensen EM. 2013b. Ultrafast endocytosis at mouse hippocampal synapses. Nature 504 :242-7.
Wenzel EM, Morton A, Ebert K, Welzel O, Kornhuber J, Cousin MA, and Groemer TW. 2012. Key physiological parameters dictate triggering of activity-dependent bulk endocytosis in hippocampal synapses. PloS One 7 :e38188.
Wucherpfennig T, Wilsch-Brauninger M, and Gonzalez-Gaitan M. 2003. Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. J Cell Biol 161:609-24.
Xue L, McNeil BD, Wu XS, Luo F, He L, and Wu LG. 2012. A membrane pool retrieved via endocytosis overshoot at nerve terminals: a study of its retrieval mechanism and role. J Neurosci 32:3398-404.
Yudowski GA, Puthenveedu MA, Henry AG, and von Zastrow M. 2009. Cargo-mediated regulation of a rapid Rab4-dependent recycling pathway. Mol Biol Cell 20 :2774-84.
Zhang B, Koh YH, Beckstead RB, Budnik V, Ganetzky B, and Bellen HJ. 1998. Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. Neuron 21 :1465-75.

Figure legends

Figure 1 – *Endocytosis modes triggered during high and low frequency stimulation in central nerve terminals.* At least two separate endocytosis modes occur in central nerve terminals and these can be revealed by labelling with a fluid phase marker such as horse radish peroxidase (HRP). Panel A shows an electron micrograph of a central nerve terminal in culture challenged with 200 action potentials delivered at 10 Hz in the presence of HRP (HRP can be visualised as an electron dense product). The majority of HRP is present inside single SVs (black arrows) indicating the dominance of CME at low stimulation frequencies. In contrast, panel B shows nerve terminals challenged with 800 action potentials at 80 Hz. Now the majority of HRP is present inside bulk endosomes (white arrows) indicating ADBE is the dominant endocytosis mode during high frequency stimulation. CME is still active under these conditions and generates single SVs (black arrows). Scale bar is equivalent to 200 nm.

Figure 2 – *Molecular steps in ADBE.* Generation of SVs via ADBE is at least a two-step process. First a bulk endosome is generated direct from the plasma membrane and second SVs are generated from the endosome. Formation of the bulk endosome is dependent on the activity-dependent dephosphorylation of dynamin I by calcineurin, an event which recruits syndapin. Dynamin I and syndapin are both required for bulk endosome formation. Equally essential is the rephosphorylation of dynamin I after stimulation by the enzymes cyclin-dependent kinase 5 (cdk5) and glycogen synthase kinase 3 (GSK3). SV generation requires the action of both adaptor protein complexes AP-1 and AP-3. Calcineurin is also required and its activity is triggered by a leak of accumulated calcium form the bulk endosome during its acidification. Once SVs are generated they join the reserve pool of SVs, a pool that is only mobilised during high intensity stimulation after prior depletion of the readily releasable pool (RRP).

Figure 3 – *Visualisation of SV generation from bulk endosomes.* The schematic shows the protocol for visualising SV budding from bulk endosomes. Primary neuronal cultures are loaded with HRP during stimulation with elevated KCl (Step 1 on schematic below). After immediate washout of HRP, neurons are stimulated to release all available SVs using 2 sequential KCl stimuli. This step depletes all HRP-labelled SVs formed via CME, leaving bulk endosomes as the only source of HRP remaining in the nerve terminal (Step 2). Cultures are then left to rest for 30 min to allow new HRP-labelled SVs to be generated from bulk endosomes (Step 3). Neurones are fixed either immediately after HRP loading (Step 1, Load), immediately after KCl unloading (Step 2, Unload) or after the 30 minute rest period (Step 3, Rest) as indicated by arrows. Representative electron micrographs are shown for the treatments described above (Steps 1, 2 and 3; Scale bar – 250 nm). Note the absence of HRP-labelled SVs after the unloading stimulus, and the appearance of new HRP-labelled SVs after the rest phase.

Figure 4 – *Endocytic recycling routes*. The fusion of newly formed endocytic vesicles with a classical sorting endosome requires the action of Rab5, early endosome antigen 1 (EEA1), syntaxin 13 (Syx13) and phosphatidylinositol-3-phosphate (PI3P). Cargo can be trafficked back to the cell surface by either a fast recycling route (requiring the combined actions of Rab4 and Rab35) or a slow recycling route via the Endosomal Recycling Compartment (ERC) (requiring the action of Rab22a and esp15 homology domain 3 (EHD3) proteins). Cargo from the ERC is then trafficked to the recycling endosome in a Rab11- and EHD1-dependent process. At this point cargo can be returned to the plasma membrane via a number of different routes. Traffic can occur via tubular extensions in an Arf6-dependent manner or via carriers (again dependent on Rab11 and EHD1). The remaining sorting endosome can mature into a lysosome via exchange of Rab5 for Rab7. This figure is not intended to be an exhaustive list of all molecules that are required for endocytic recycling, but rather highlights those discussed in this review. For a comprehensive list please consult (Grant and Donaldson 2009).

Figure 5 – *Potential utilisation of the endocytic recycling machinery by ADBE*. ADBE may utilise the endocytic recycling pathway at a variety of stages. First SV cargo may traffic via a sorting endosome after the production of carrier vesicles from the bulk endosome in an AP-1- and Rab5-dependent process. Mature SVs can then be generated from the sorting endosome by AP-3. Alternatively the bulk endosome may fuse directly with the sorting endosome in a calcineurin-dependent manner. This event would be triggered by bulk endosome acidification and local efflux of accumulated calcium. Finally plasma membrane cargo may be recycled to the cell surface either via the slow recycling pathway (Rab11-dependent) or alternatively be degraded via the lysosomal system (Rab7-dependent).

Box 1 – Cargo sorting and trafficking during endocytic recycling

Endocytic recycling is required to ensure the correct molecular composition of the plasma membrane and specific intracellular compartments. At the nerve terminal, the most important compartment is the synaptic vesicle (SV), which releases stored neurotransmitter after its fusion with the plasma membrane during action potential stimulation. During CME the correct SV cargo are clustered together at the plasma membrane by the adaptor protein complex AP-2, which recognizes and binds to specific motifs present on their cytoplasmic domains. Not all SV cargoes possess such motifs, and this cargo is clustered via interactions with both other SV cargo and monomeric adaptor proteins such as AP180 and stonin. Since SVs are formed directly at the plasma membrane it is vital that this process of cargo sorting is highly efficient, since there is no other processing step. Thus very few presynaptic plasma membrane molecules are accumulated during SV formation by CME.

Classical endocytic recycling by definition requires the uptake of extracellular ligands which are clustered by binding to plasma membrane receptors. In its simplest form, endocytic recycling exists

to allow the uncoupling of extracellular cargo from accumulated plasma membrane receptors and then their subsequent trafficking either for degradation (for extracellular cargo) or recycling back to the cell surface (for the plasma membrane receptors). In addition endocytic recycling exists to return accumulated membrane to the cell surface in addition to the receptors themselves, since this is essential for membrane homeostasis. The endocytic recycling system can also be dynamically modulated to alter either the location or concentration of specific plasma membrane cargo, such as transporters or receptors. Thus endocytic recycling is essential for accumulation and processing of essential nutrients, maintaining the integrity of the plasma membrane and finally dynamically modulating the ability of the cell to respond to its environment by regulating the composition of plasma membrane cargo molecules.