The Molecular Physiology of Activity-Dependent Bulk Endocytosis of Synaptic Vesicles

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Abstract

Central nerve terminals release neurotransmitter in response to a wide variety of stimuli. Since maintenance of neurotransmitter release is dependent on the continual supply of synaptic vesicles (SVs), nerve terminals possess an array of endocytosis modes to retrieve and recycle SV membrane and proteins. During mild stimulation conditions single SV retrieval modes such as clathrin-mediated endocytosis (CME) predominate. However during increased neuronal activity additional SV retrieval capacity is required, which is provided by activity-dependent bulk endocytosis (ADBE). ADBE is the dominant SV retrieval mechanism during elevated neuronal activity. It is a high capacity SV retrieval mode that is immediately triggered during such stimulation conditions. This review will summarise the current knowledge regarding the molecular mechanism of ADBE, including molecules required for its triggering and subsequent steps, including SV budding from bulk endosomes. The molecular relationship between ADBE and the SV reserve pool will also be discussed. It is becoming clear that an understanding of the molecular physiology of ADBE will be of critical importance in attempts to modulate both normal and abnormal synaptic function during intense neuronal activity.

Keywords

Endocytosis; synaptic vesicle; calcineurin; dynamin; clathrin; activity

SV retrieval capacity during intense nerve terminal stimulation

Central nerve terminals are small subcellular compartments that contain typically 100-200 synaptic vesicles (SV). This pool of SVs is limited and thus to maintain the fidelity of neurotransmission SV membrane and protein must be retrieved and recycled very efficiently after exocytosis. This is particularly important during times of intense stimulation, since excess SV membrane will be inserted into the plasma membrane and will dangerously increase surface area in a short period of time. Therefore it is essential that nerve terminals have SV retrieval mechanisms to accommodate sudden and large changes in the plasma membrane composition during intense stimuli.

Clathrin-mediated endocytosis (CME) retrieves single SVs *de novo* from the nerve terminal plasma membrane using the coat protein clathrin and various adaptor proteins including AP-2, epsin and AP180 (Edeling et al. 2006). It is the best characterised SV retrieval route, mainly due to parallel molecular studies of clathrin-dependent endocytosis pathways in non-neuronal cells. CME is highly accessible to molecular perturbation in such systems by either overexpression of dominant negative proteins or knockdown of protein expression by RNAi

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(Jung and Haucke 2007;Ungewickell and Hinrichsen 2007). This has allowed the exploitation of numerous mutants in neurones that have been previously characterised in non-neuronal cells. A summary of the molecular mechanism of CME is outwith the scope of this manuscript, but can be found in many review articles (Brodin et al. 2000;Slepnev and De Camilli 2000;Edeling et al. 2006;Ungewickell and Hinrichsen 2007;Doherty and McMahon 2009).

Another SV retrieval mode is "kiss-and-run" where the SV never fully fuses with the plasma membrane during exocytosis and retrieves intact. The existence of kiss-and-run in large secretory cells was demonstrated using simultaneous high resolution capacitance and amperometry measurements (Alés et al. 1999). Kiss-and-run also occurs in large atypical central nerve terminals, since transient (less than 2 sec) single SV fusion events were observed using cell-attached capacitance measurements at release sites (He et al. 2006). The existence of kiss-and-run has been more difficult to definitively prove in typical central nerve terminals due to their small size. A number of indirect fluorescent techniques have been employed to prove the occurrence of kiss-and-run (Klingauf et al. 1998;Aravanis et al. 2003;Gandhi and Stevens 2003;Harata et al. 2006), however recent studies examining the uptake and differential release of fluorescent nanoparticles provide to best current evidence for its existence (Zhang et al. 2009). Little is known about the molecular mechanism of this SV recycling mode, since more direct methods to monitor its modulation have only recently been reported. However with the advent of these methods rapid progress should be made in elucidating key molecules in this process.

Both CME and kiss-and-run involve the internalisation of single SVs. During mild stimulation very few SVs fuse with the nerve terminal plasma membrane, meaning these SV retrieval modes can cope with the retrieval demand. When the nerve terminal is challenged with more intense stimuli however, it requires additional capacity to retrieve the extra SV membrane and proteins inserted into the plasma membrane. Additional retrieval capacity could be achieved in two different ways; first either CME or kiss-and-run could operate at increased rates, or second another SV endocytosis mode could be recruited to aid retrieval. The first option is unlikely, since a number of groups have demonstrated that CME has a maximal rate (time constant of approximately 15 sec) that does not scale with stimulation intensity (Jockusch et al. 2005;Granseth et al. 2006;Balaji and Ryan 2007) (but see (Zhu et al. 2009)). Thus CME operates in a similar way to an enzyme, with a fixed rate but limited capacity, resulting in the saturation of this SV retrieval mode during intense nerve terminal stimulation (Sankaranarayanan and Ryan 2000). Intense stimulation may increase the speed of kiss-and-run, since there is evidence of a similar mode of SV retrieval (rapid endocytosis) being activated by strong stimulation in large atypical nerve terminals (Beutner et al. 2001;Wu et al. 2005). This suggests that kiss-and-run may be able to partially support the transient demand for SV membrane retrieval during intense stimulation.

Both CME and kiss-and-run are active in typical small central nerve terminals during intense stimulation (Granseth et al. 2006;Zhang et al. 2009). However due to their low capacity, both SV retrieval modes will be unable to fully compensate for the large increases in nerve terminal surface area that occur during periods of elevated neuronal activity. Therefore another SV retrieval mode must be recruited to increase endocytic capacity during periods of intense activity in nerve terminals. That retrieval mode is activity-dependent bulk endocytosis (ADBE).

ADBE is a fast, high capacity SV retrieval mechanism

ADBE was first reported in the amphibian neuromuscular junction (Miller and Heuser 1984) and since then has been observed in numerous neuromuscular preparations from other

organisms and in both typical and atypical central nerve terminals. ADBE is typically characterised by large invaginations of plasma membrane which then fission to form endosomal-like compartments. SVs can then bud from these bulk endosomes to rejoin the pool of fusion competent SVs. Since ADBE can internalise large areas of plasma membrane, it has the potential capacity to correct for significant changes in nerve terminal surface area during increased neuronal activity. For this SV retrieval mode to be physiologically relevant however, it must be triggered by intense stimuli and have suitable kinetics to rapidly reverse the increase in nerve terminal surface area caused by the insertion of SV membranes. ADBE fulfils both of these criteria (reviewed more fully in (Cousin 2009)), implicating it as the dominant SV retrieval mode during increased neuronal activity in central nerve terminals.

Many studies have shown that ADBE is activated in either central nerve terminals or neuromuscular preparations by large non-physiological stimuli (Miller and Heuser 1984;Marxen et al. 1999;Leenders et al. 2002;de Lange et al. 2003;Holt et al. 2003;Di Paolo et al. 2004;Evans and Cousin 2007). However recent studies in both preparations have shown that this SV retrieval mode can also be triggered by trains of high intensity action potentials within the physiological range (Richards et al. 2000;Teng and Wilkinson 2000;Paillart et al. 2003;Richards et al. 2003;Wu and Wu 2007;Clayton et al. 2008;Clayton et al. 2009). As its name suggests, ADBE is specifically triggered during elevated neuronal activity in central nerve terminals (Harata et al. 2001; Andersson et al. 2008;Clayton et al. 2008). This triggering occurs co-incident with increased neuronal activity in neuromuscular preparations (Miller and Heuser 1984;Teng et al. 2007) and in typical (Marxen et al. 1999;Leenders et al. 2002;Clayton et al. 2008) and atypical central nerve terminals (Holt et al. 2003;Paillart et al. 2003;Wu and Wu 2007;Wu et al. 2009), indicating that ADBE will be an active and key participant in SV retrieval during these stimulation conditions. ADBE also swiftly terminates when this stimulus is removed (Miller and Heuser 1984;Wu and Wu 2007;Clayton et al. 2008), in contrast to CME which persists for minutes after intense stimulation terminates (Miller and Heuser 1984;Clayton et al. 2008). Thus the triggering of ADBE is rapid and is only active during elevated neuronal stimuli.

The addition of ADBE to the array of different endocytosis modes available to the nerve terminal provides it with the capability to handle an extremely wide range of stimuli. During mild stimulation when few SVs are fusing with the plasma membrane, the dominant SV retrieval mode is CME, since the demand for plasma membrane retrieval is small (Granseth et al. 2006;Zhu et al. 2009). However when neuronal activity increases, CME lacks the capacity to deal with the additional retrieval demands placed on the nerve terminal, and ADBE is triggered. After cessation of stimulation ADBE terminates, leaving CME to revert to being the dominant SV retrieval mode in the nerve terminal.

Visualisation and monitoring of ADBE

Investigations into both the physiology and molecular mechanism of ADBE have employed a combination of experimental approaches to monitor this SV retrieval mode in both living neurones and fixed tissue. To date electron microscopy has remained the most unambiguous method of monitoring ADBE. Large cisternae and/or endosomes are routinely observed in either neuromuscular preparations or central neurons that were fixed during intense stimulation using this approach (Miller and Heuser 1984;Takei et al. 1996;Gad et al. 1998). These structures were absent during either resting or mild stimulation conditions. Various groups have confirmed that such structures are endocytic, by applying external markers to label either the fluid or membrane phase. The most common fluid phase marker is horse radish peroxidase (HRP) which can be converted to an electron dense product. HRP labels all active SV retrieval modes, providing a useful comparison of parallel endocytic routes such as CME (Marxen et al. 1999;Leenders et al. 2002;Di Paolo et al. 2004;Evans and

Cousin 2007;Clayton et al. 2008;Clayton et al. 2009). A selective labelling of ADBE can also be achieved at the morphological level by employing fluid phase markers such as ferritin, which are too large to be retrieved inside single SVs (Paillart et al. 2003). Membrane markers such as the dye FM1-43 can also be used to monitor both ADBE and CME in morphological studies, since they can be photoconverted into electron dense products (Richards et al. 2000). However the efficiency of photoconversion seems to vary dependent on the shape of the endosome, with dye in closely opposed cisternae demonstrating more efficient conversion than inside large round endosomes (Richards et al. 2000;Teng and Wilkinson 2000;de Lange et al. 2003;Akbergenova and Bykhovskaia 2009;Vijayakrishnan et al. 2009).

Electrophysiological approaches have also been used to demonstrate that large invaginations fission directly from the plasma membrane to form bulk endosomes (Wu and Wu 2007;He et al. 2009;Wu et al. 2009). This key finding complements morphological studies, since HRP-labelled endosomes could also in theory be generated by the fusion of single HRP-labelled SVs with a sorting endosome (Rizzoli et al. 2006). Using capacitance measurements in the Calyx of Held, large downward changes were observed that were much larger than would be expected from retrieval of a single SV (Wu and Wu 2007). In addition a fission pore could also be detected using this approach (Wu and Wu 2007). Similar observations have also been reported using direct cell-attached recordings at the release sites of this atypical nerve terminal (He et al. 2009).

ADBE can also be monitored in cultured neurones in real time using fluorescent assays. The simplest method is to monitor the uptake of large inert fluorescent fluid phase markers. One such marker, dextran, is too large to label single SV endocytosis modes and therefore provides a selective report of ADBE. This approach has been used in various neuronal preparations including lizard neuromuscular junctions (Teng et al. 2007), retinal bipolar neurones (Holt et al. 2003) and primary cultures of central neurones (Clayton et al. 2008;Clayton et al. 2009).

Another method to monitor ADBE in real time is to use styryl dyes such as FM1-43 and FM2-10. These dyes possess a hydrocarbon tail that allows them to partition into membranes and a quaternary nitrogen head group that disallows their passage though the bilayer (Betz et al. 1996). Importantly these dyes only fluoresce when partitioned in membrane, making them excellent reporters of actively recycling SVs (Cousin and Robinson 1999). Although these dyes should label any retrieving membrane non-specifically, a discrete labelling of different endocytic modes has been observed by a number of groups (Richards et al. 2000;Virmani et al. 2003;Richards et al. 2004;Evans and Cousin 2007;Clayton et al. 2008;Clayton and Cousin 2008). This was first observed at the amphibian neuromuscular junction, by loading the dyes FM1-43 and FM2-10 during tetanic stimulation. Photoconversion of FM1-43-loaded nerve terminals showed dye in both single SVs and large cisternae, whereas FM2-10 preferentially labelled single SVs (Richards et al. 2000). The selective labelling of ADBE by FM1-43 is also observed in central nerve terminals. This phenomenon has been confirmed by a number of criteria. For example an activity-dependent increase in FM1-43 loading, but not FM2-10, occurs during stimuli that trigger ADBE (Clayton and Cousin 2008). Also a selective arrest of FM1-43 loading, but not FM2-10, was observed after molecular manipulations that selectively blocked ADBE (Evans and Cousin 2007;Clayton et al. 2009). These manipulations had no effect on FM1-43 uptake evoked by mild stimulation, confirming the selective labelling of ADBE by this dye during elevated neuronal activity (Evans and Cousin 2007;Clayton et al. 2009).

Why does FM1-43, but not FM2-10, selectively label ADBE? A number of theories have been proposed. The original explanation was that ADBE was a very slow process that

persisted for minutes after stimulation. Since slowly invaginating bulk endosome cisternae would still be connected to the extracellular space, it was argued that the more hydrophilic FM2-10 was washed out of these structures whereas FM1-43 was retained (Richards et al. 2000). However this explanation conflicts with recent studies showing that ADBE is rapid and terminates immediately after stimulation (Cousin 2009). An alternative explanation for the selective labelling of ADBE by FM1-43 could be the affinity of this dye for bulk endosome membrane. Supporting evidence comes from the fact that FM1-43 and FM2-10 labelling of this SV retrieval mode can be manipulated by changing their loading concentration, but not the duration of dye incubation (Clayton and Cousin 2008). The molecular basis for the differential affinity of these dyes for bulk endosome membrane is still unclear. One possibility is that bulk endosome membrane has a different lipid microenvironment to that of the plasma membrane. In support a number of fluid phase uptake routes that are related to ADBE have an enrichment of lipid rafts (Doherty and McMahon 2009). Possible differences in lipid composition between bulk endosome membranes generated by ADBE and SVs generated by CME may also contribute towards a heterogenous population of SVs within nerve terminals, possibly explaining the selective repopulation of specific SV pools by both ADBE and CME (Richards et al. 2000;Evans and Cousin 2007;Clayton et al. 2009).

Molecular mechanism of ADBE

Very little is known about the molecular mechanism of ADBE, despite the fact that it was identified over thirty years ago. Recently progress has been made in identifying key molecules in this mode of SV retrieval, which should serve as excellent starting points for further investigation.

Triggering of ADBE by calcineurin

Since ADBE is triggered immediately by increases in neuronal activity, it suggests there is a molecular sensor that detects such changes. One possible triggering mechanism is saturation of the CME machinery, however this may not provide the temporal resolution observed in physiological studies (Teng et al. 2007;Wu and Wu 2007;Clayton et al. 2008). The ideal trigger for ADBE would accurately report both the extent and duration of neuronal activity. One such trigger could be calcium influx.

For calcium influx to be the molecular trigger for ADBE, a sensor molecule must be present to detect changes in calcium within the nerve terminal. The synaptotagmin family of proteins are widely accepted to be the calcium sensor for SV exocytosis (Rizo and Rosenmund 2008;Sudhof and Rothman 2009). These proteins also have roles in CME, principally clustering SV proteins with the clathrin adapter protein AP-2 (Haucke and De Camilli 1999). This interaction is calcium-independent however (Zhang et al. 1994), and other studies have shown that the calcium binding ability of synaptotagmin plays no role in SV endocytosis (Cousin and Robinson 1998;Marks and McMahon 1998).

Recent electrophysiological studies have shown that ADBE is both calcium- and calmodulin-dependent (Wu et al. 2009). There is now strong evidence that the calcium- and calmodulin-dependent protein phosphatase calcineurin is the activity-dependent trigger for ADBE. Calcineurin co-ordinately dephosphorylates at least eight different substrates on prolonged depolarization of central nerve terminals (the dephosphins), all of which have essential roles in SV endocytosis (Cousin and Robinson 2001;Lee et al. 2005). Evidence that calcineurin-dependent dephosphorylation events are the trigger for ADBE have been accumulating. Firstly calcineurin has a low micromolar affinity for calcium (Klee et al. 1979). This is important since calcineurin has a cytosolic location, meaning the enzyme would only encounter sufficient intracellular calcium levels for its activation during intense

neuronal activity. Also an activity-dependent requirement for calcineurin in SV endocytosis has been demonstrated in different neuronal systems using pharmacological antagonists of the enzyme (Chan and Smith 2001;Kumashiro et al. 2005;Evans and Cousin 2007). However critical evidence for the role of calcineurin as the trigger for ADBE came from the demonstration that the dephosphorylation of one if its key substrates, the large GTPase dynamin I, only occurred at the same stimulation conditions that triggered this SV retrieval mode (Clayton et al. 2009). Since calcineurin activation had the same activation threshold as ADBE, it implicated its activity-dependent dephosphorylation of the dephosphins as a key step in this SV retrieval mode.

Dynamin I dephosphorylation

Dynamin has three isoforms, all of which are expressed in nerve terminals, with dynamin I having the highest level of expression (Cao et al. 1998). The GTPase activity of dynamin is essential for vesicle fission during CME in non-neuronal systems (Marks et al. 2001). This activity is also essential for CME in central nerve terminals, since its inhibition by the antagonist dynasore (Macia et al. 2006) abolished SV retrieval during mild stimulation of primary neuronal cultures (Newton et al. 2006). Dynamin GTPase activity is also essential for ADBE, since dynasore equally inhibited the generation of both SVs and bulk endosomes during high frequency action potential stimulation in the nerve terminals of primary neuronal cultures (Clayton et al. 2009)

Multiple lines of evidence have shown that dynamin I dephosphorylation is also essential for ADBE in central nerve terminals in addition to a requirement for its GTPase activity. Firstly dynamin I is only dephosphorylated by calcineurin during neuronal activity that triggers ADBE (Clayton et al. 2009). Secondly, inhibition of cyclin-dependent kinase 5 (the protein kinase that rephosphorylates dynamin I after stimulation (Tan et al. 2003)) selectively arrests ADBE, but has no effect on CME (Evans and Cousin 2007). Thirdly, overexpression of dominant negative dynamin I containing mutations in its major phosphorylation sites, arrested the uptake of FM1-43, but not FM2-10 during trains of high frequency action potentials (Clayton et al. 2009). Finally defects in SV endocytosis were only observed during strong but not mild neuronal activity in primary cultures derived from dynamin I knockout mice (Ferguson et al. 2007). These defects were only observed during intense stimulation, not afterwards (Ferguson et al. 2007). Since ADBE is the dominant SV retrieval mode during, but not after, intense stimulation, this implicates calcineurin-dependent dynamin I dephosphorylation as a key event in this SV retrieval pathway.

The assertion that activity-dependent dynamin I dephosphorylation is the key trigger for ADBE has been questioned by recent data from the dynamin I knockout mouse. Firstly HRP-labelled bulk endosomes were observed in some inhibitory nerve terminals of knockout cultures during KCl stimulation (Hayashi et al. 2008), suggesting ADBE could occur without dynamin I in certain nerve terminal subtypes. Secondly, electrophysiological studies in Calyx of Held synapses derived from dynamin I knockout mice showed a selective retardation of a slow, post-stimulation mode of SV endocytosis, whereas a fast mode that was triggered during stimulation was similar to wild-type (Lou et al. 2008). These results directly contradict earlier results observed in neuronal cultures from these mice, where knockout of dynamin I ablated a fast activity-dependent SV endocytosis mode (Ferguson et al. 2007). One possible reason for these contradictory findings is redundancy between different isoforms of dynamin. For example dynamin III is dephosphorylated on similar sites to dynamin I during nerve terminal depolarization (Graham et al. 2007) and may compensate for the loss of dynamin I during ADBE. Gene ablation or RNAi studies that arrest expression of other dynamin isoforms may therefore resolve these contrasting results. However the current balance of evidence is that both dynamin I GTPase activity and its dephosphorylation are essential for ADBE.

Syndapin

Dynamin I interacts with a number of proteins that are essential for SV endocytosis via its proline-rich C-terminus, including amphiphysin, endophilin and syndapin (Grabs et al. 1997;Ringstad et al. 1997;Qualmann et al. 1999). However the only phosphorylation-dependent interaction is with syndapin I, where dynamin I dephosphorylation allows their interaction (Anggono et al. 2006). Since syndapin only interacts with dynamin I when it is dephosphorylated it suggests that this association may be critical for ADBE. In agreement ADBE is arrested by either overexpression of dominant negative dynamin I mutants that do not interact with syndapin (Anggono and Robinson 2007) or delivery of competitive peptides that disrupt the endogenous interaction in neuronal cultures (Clayton et al. 2009). Importantly disruption of the dynamin - syndapin interaction had no effect on CME in the same system, suggesting a selective requirement for syndapin I in ADBE. In support, knockdown of syndapin I with shRNA vectors abolished ADBE in primary neuronal cultures (Clayton et al. 2009). Furthermore anti-syndapin antibodies injected into invertebrate nerve terminals selectively arrested ADBE but had no effect on CME (Andersson et al. 2008).

How could syndapin I control ADBE in nerve terminals? Syndapin has a similar structure to other dynamin I binding partners such as amphiphysin and endophilin, with all three having an N-terminal bin-amphiphysin-RVS (BAR) domain and a C-terminal dynamin I-binding src-homology 3 (SH3) domain (Grabs et al. 1997;Ringstad et al. 1997;Qualmann et al. 1999). One difference however is that syndapin has an F-BAR domain, in comparison to the N-BAR domains of both amphiphyin and endophilin (Peter et al. 2004;Itoh et al. 2005). BAR domains sense membrane curvature and can also tubulate membrane *in vitro* and *in vivo* (McMahon and Gallop 2005;Dawson et al. 2006). N-BAR domains associate with membranes of very tight curvature very similar to that of a SV (Peter et al. 2004), implicating them in endocytosis modes that retrieve single SVs. In contrast F-BAR domains associate with membranes of much more shallow curvature (Henne et al. 2007;Shimada et al. 2007), suggesting that syndapin could be recruited to large invaginations during ADBE. Therefore the curvature of invaginating bulk endosomes could act as a coincidence detector for syndapin I recruitment in concert with dynamin I dephosphorylation (Fig 1). Whether syndapin plays an active role in driving either curvature, tubulation and/or fission of bulk invaginations remains to be addressed. Regardless, syndapin I has emerged as a key player in ADBE in central nerve terminals and further studies will elucidate its exact molecular mechanism.

Molecular mechanism for the triggering of ADBE

The essential requirement for the activity-dependent dephosphorylation of dynamin I and its subsequent interaction with syndapin I has provided the first mechanistic insight into the molecules that control ADBE. It also suggests that the activity-dependent dephosphorylation of dynamin I is the key event in the selection of SV retrieval modes in the nerve terminal. A model of how this could occur is described below (Fig 1). During mild neuronal activity, dynamin I is not dephosphorylated (Clayton et al. 2009) therefore ADBE is not activated. This agrees with studies showing CME is the major membrane SV retrieval mode during these stimulation conditions (Granseth et al. 2006;Clayton et al. 2008;Zhu et al. 2009). Dynamin I has an essential interaction with the N-BAR protein amphiphysin during CME endocytosis (Shupliakov et al. 1997;Jockusch et al. 2005). Importantly, dynamin I binds amphiphysin irrespective of its phosphorylation status (Tan et al. 2003;Anggono et al. 2006;Graham et al. 2007) potentially explaining why CME can proceed independent to the level of neuronal activity (Fig 1). Thus CME is dynamin-dependent, but not dependent on dynamin I dephosphorylation. When nerve terminals are challenged by stronger stimulation, the activity-dependent increase in calcium influx stimulates calcineurin to dephosphorylate

dynamin I. This dephosphorylation event then permits the interaction of dynamin I with syndapin to trigger ADBE (Fig 1). Thus while dynamin I GTPase activity is essential for all modes of SV endocytosis from the nerve terminal plasma membrane, its dephosphorylation is only required for ADBE.

Actin dynamics

Syndapin I can also interact with the neuronal Wiskott-Aldrich syndrome protein (N-WASP) via its SH3 domain (Kessels and Qualmann 2002). N-WASP recruits the arp2/3 complex to nucleate actin polymerisation (Takenawa and Miki 2001), suggesting syndapin I may also coordinate actin dynamics during ADBE (Kessels and Qualmann 2002). Syndapin I can bind dynamin I and N-WASP simultaneously, since it can form homo-oligomers via its F-BAR domain (Kessels and Qualmann 2006;Halbach et al. 2007). This means that syndapin I complexes that are recruited to sites of ADBE by dynamin I may also recruit N-WASP to promote actin polymerisation. In support syndapin I antibodies reduced actin accumulation at endocytic sites when injected into invertebrate nerve terminals (Andersson et al. 2008).

Actin polymerisation is already implicated in ADBE, since this SV retrieval mode shares many similarities with the process of macropinocytosis (Royle and Lagnado 2003) which is the uptake of extracellular fluid during membrane ruffling in non-neuronal cells (Nichols and Lippincott-Schwartz 2001;Conner and Schmid 2003;Doherty and McMahon 2009). Macropinocytosis is driven by the dynamic rearrangement of the actin cytoskeleton which causes the closure of lamellapodia to create large internal vesicles termed macropinosomes. While the molecular mechanisms that control macropinocytosis are still poorly understood, both Rho family GTPases and phosphatidylinositol 3-kinase (PI3K) have essential roles in the control of actin dynamics for this process (Nichols and Lippincott-Schwartz 2001;Conner and Schmid 2003;Doherty and McMahon 2009). Intriguingly both actin dynamics and PI3K activity are essential for ADBE in both amphibian neuromuscular junctions and retinal bipolar neurones (Holt et al. 2003;Richards et al. 2004), suggesting that actin turnover may also be critical for this SV retrieval mode.

Lipid metabolism

The identification of PI3K as a possible key enzyme in ADBE highlights an emerging role for lipid metabolism in this SV retrieval mode. Interestingly a number of enzymes that control the levels of a specific phospholipid, phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$) have been implicated in ADBE. Synaptojanin is one such enzyme. Synaptojanin is the major lipid phosphatase in nerve terminals (Cremona et al. 1999) and has two catalytic domains, a $5'$ phosphatase domain (that specifically cleaves phosphate from the $5'$ position of the inositol ring) and a sac-1 domain (that has a less specific inositol phosphatase activity) (Guo et al. 1999;Tsujishita et al. 2001). Cultures derived from synaptojanin knockout mice have defects in SV endocytosis at all stimulation intensities, suggesting a universal role for this enzyme in all SV retrieval modes (Mani et al. 2007). However synaptojanin could not rescue SV endocytosis during intense stimulation in knockout cultures when its $5'$ phosphatase domain was mutated, whereas synaptojanin containing sac-1 mutations could (Mani et al. 2007). This suggests an essential activity-dependent requirement for the 5-phosphatase activity of synaptojanin in SV endocytosis, implicating it in ADBE.

The major protein interaction partner of synaptojanin is endophilin (Micheva et al. 1997;Ringstad et al. 1997) with studies in model organisms suggesting that endophilin recruits synaptojanin to sites of endocytosis (Schuske et al. 2003;Verstreken et al. 2003). When synaptojanin mutants that could not bind endophilin were overexpressed in knockout cultures, SV endocytosis was only rescued during mild neuronal activity (Mani et al. 2007). This suggests that the endophilin-synaptojanin interaction is also critical for ADBE.

Intriguingly both synaptojanin phosphatase activity and its interaction with endophilin are controlled its phosphorylation status (Lee et al. 2004). Synaptojanin is a dephosphin, and is dephosphorylated by calcineurin during prolonged stimulation (McPherson et al. 1994;Marks and McMahon 1998). Thus synaptojanin dephosphorylation provides a attractive mechanism for both its recruitment to endocytic sites (via endophilin) and its modification of membrane phospholipids (via it 5-phosphatase activity) during ADBE.

To further highlight the possible key role played by $PI(4,5)P_2$ in ADBE, the major PIP kinase at the synapse, phosphatidylinositol (4) phosphate 5 kinase type I γ (PIPKIγ) (Di Paolo et al. 2004) is also dephosphorylated by calcineurin during prolonged nerve terminal stimulation (Lee et al. 2005). This dephosphorylation permits an interaction with talin, which activates the enzyme, increasing $PI(4,5)P_2$ levels in the nerve terminal (Lee et al. 2005). Thus calcineurin-dependent dephosphorylation is critical in regulating both the synthesis and metabolism of $PI(4,5)P_2$ at the synapse. Future experiments will determine whether both PIPKI(and synaptojanin are dephosphorylated in a similar activity-dependent manner to dynamin I and whether these events are essential for ADBE.

The importance of $PI(4,5)P_2$ metabolism in ADBE was reenforced by the discovery of the integral membrane lipase Rolling Blackout in *Drosophilia* (Huang et al. 2004). Temperature-sensitive mutants displayed paralysis within minutes and an activity-dependent accumulation of $PI(4,5)P_2$ with a concomitant depletion of diacylglycerol (Huang et al. 2004). This temperature-sensitive mutation also inhibited the uptake of FM1-43 during strong stimulation at both the neuromuscular junctions and central nerve terminals of these flies (Vijayakrishnan et al. 2009). FM1-43 photoconversion studies at the neuromuscular junction demonstrated that generation of bulk endosomes was inhibited during strong stimulation at the restrictive temperature. In contrast the number of SVs containing FM1-43 concomitantly increased, suggesting an attempted compensation by CME (Vijayakrishnan et al. 2009).

Thus the control of $PI(4,5)P_2$ levels within the nerve terminal seems to have a key role in ADBE. How $PI(4,5)P_2$ exerts its function is still unknown but may possibly involve recruitment of proteins with specific $PI(4,5)P_2$ interaction domains (Doherty and McMahon 2009) or alternatively by regulating the interaction of the cytoskeleton with the plasma membrane (Raucher et al. 2000). $PI(4,5)P_2$ synthesis and metabolism is also essential for CME (Doherty and McMahon 2009). For example, $PI(4,5)P_2$ generation is a critical co-incidence detector for the recruitment of clathrin adapter proteins, whereas its breakdown is a key step in the uncoating of a SV (Takei and Haucke 2001;Jung and Haucke 2007). Since both CME and ADBE require $PI(4,5)P_2$, it suggests that the temporal and spatial production of this phospholipid will be critical in the differential control of both SV retrieval modes. In this regard the calcineurin-dependent dephosphorylation of both PIPKI(and synaptojanin may be key determinants in the triggering and localisation of $PI(4,5)P_2$ production specifically for ADBE.

Other implicated proteins

In addition to the proteins mentioned above other molecules have been implicated in ADBE. For example overexpression of different splice variants of the plasma membrane-localised synaptotagmin 7 result in the recruitment of different SV endocytosis modes (Virmani et al. 2003). Overexpression of a synaptotagmin 7A variant containing both C2 domains shifted the mode of SV endocytosis towards ADBE, whereas the truncated 7B form accelerated CME (Virmani et al. 2003). In a different study the classical AP-3-dependent endosomal recycling pathway has been implicated in SV endocytosis activated by high intensity stimulation. This trafficking route involves the fusion of single SVs with sorting endosomes before a budding event dependent on the adaptor protein AP-3 (Newell-Litwa et al. 2007).

In these studies, the recycling of the glutamate transporter VGLUT was switched from CME to an AP-3-dependent mode during high intensity stimulation (Voglmaier et al. 2006).

Molecular mechanism of SV budding from bulk endosomes

One of the key aspects of CME is the clustering and sorting of cargo at the plasma membrane prior to internalisation. Evidence that SV proteins are sorted at this level comes from studies showing that at least 4 independent integral SV proteins have very similar internalisation kinetics during stimuli which only activate CME (Diril et al. 2006;Granseth et al. 2006;Voglmaier et al. 2006;Ferguson et al. 2007;Kim and Ryan 2009). These proteins are thought to cluster due to endocytic recognition motifs on their cytoplasmic domains for the clathrin adapter protein AP-2 (Zhang et al. 1994;Haucke and De Camilli 1999). However what process ensures that SV cargo is internalised by ADBE? Parallel studies of macropinocytosis in non-neuronal cells have revealed very little active accumulation of cargo at the level of the plasma membrane. However there does seem to be an exclusion of specific proteins, suggesting membrane micro-environments such as lipid rafts may play an initial role in cargo sorting (Mercanti et al. 2006).

It is most likely that clustering and enrichment of protein cargo into SVs occurs not at the plasma membrane, but rather during SV budding from the bulk endosome. This implicates clathrin in this event, since sorting of SV cargo requires clathrin adaptor proteins. This is supported by a number of independent studies showing clathrin coats on SVs budding from bulk endosomes in either central nerve terminals (Takei et al. 1996), invertebrate nerve terminals (Gad et al. 1998;Andersson et al. 2008) or the lizard neuromuscular junction (Teng and Wilkinson 2000). Furthermore acute inactivation of either the clathrin light or heavy chain in *Drosophila* nerve terminals resulted in the accumulation of large membranous cisternae that could not produce SVs for reentry into the recycling pool (Heerssen et al. 2008;Kasprowicz et al. 2008) (but see (Sato et al. 2009)). The identity of the adapter proteins that mediate clathrin-dependent SV budding is still unknown, however the most likely would seem to be AP-3, since this pathway produces functional SVs for exocytosis (Scheuber et al. 2006) and is required for SV recycling during intense stimulation in central nerve terminals (Voglmaier et al. 2006).

It is likely that dynamin I will be required for the fission of SVs from bulk endosomes, however there are other alternatives. The eps15 homology domain (EHD) family of proteins (EHDs/RME-1/pincher) have been implicated in various forms of clathrin-independent endocytosis and endosomal traffic (Grant et al. 2001;Lin et al. 2001;Shao et al. 2002). Recent structural studies have shown that these proteins belong to the dynamin superfamily (Daumke et al. 2007) suggesting they could mediate SV fission. Intriguingly syndapin interacts with the EH domain of the EHD proteins via its NPF repeats, providing a molecular link between the creation of bulk endosomes from plasma membrane and the generation of SVs from bulk endosomes (Braun et al. 2005). Further studies will be required to determine the role of EHD proteins in SV fission from bulk endosomes and their regulation by syndapin interactions.

Molecular and physiological links between ADBE and the reserve SV pool

SVs in nerve terminals can be arbitrarily divided into three notional pools, the RRP, the recycling pool and reserve pool (Sudhof 2000;Rizzoli and Betz 2005). However the interpretation of which SVs populate each pool relies very much on the viewpoint of individual researchers. Traditionally the reserve pool of SVs in central nerve terminals is proposed to be only mobilized during neuronal activity that exceeds typical stimulation conditions (Rizzoli and Betz 2005), suggesting there may be a functional link between this event and ADBE. This is indeed the case, with ADBE proposed to provide SVs that

selectively repopulate the reserve SV pool. The first evidence for this came from studies in the amphibian neuromuscular junction. In these studies SVs that were labelled with FM2-10 (which does not label ADBE) during tetanic stimulation were almost immediately available for fusion, whereas those labelled with FM1-43 (which does label ADBE) were unable to fuse for at least 10 minutes (Richards et al. 2000). Furthermore reserve pool replenishment was blocked by perturbation of ADBE with either actin destabilising agents or PI3K inhibitors (Richards et al. 2004). A similar phenomenon is also observed in central nerve terminals, where SVs generated by intense stimulation support a sustained phase of exocytosis (Evans and Cousin 2007;Clayton et al. 2009). These SVs were generated by ADBE, since a variety of manipulations that inhibit this SV retrieval mode eliminate the subsequent sustained component of SV fusion (Evans and Cousin 2007;Clayton et al. 2009). Thus ADBE selectively replenishes the reserve pool of SVs, a pool that is only mobilised to support neurotransmitter release during elevated neuronal activity.

The fact that ADBE and mobilisation of reserve pool SVs are triggered by very similar stimuli suggests that they may share either overlapping or similar molecules in their mechanism. This appears to be true, with essential roles for reserve SV pool maintenance or mobilisation proposed for both actin (Hilfiker et al. 1999;Shupliakov 2009) and PI3K (Cousin et al. 2003). Furthermore the SV protein synapsin (which is proposed to control the size of the reserve SV pool (Hilfiker et al. 1999)) is dephosphorylated on multiple sites by calcineurin (Jovanovic et al. 2001;Chi et al. 2003) and rephosphorylated by cyclin-dependent kinase 5 and MAP kinase (Jovanovic et al. 1996;Matsubara et al. 1996). This dephosphorylation event only occurs during strong stimulation (Chi et al. 2003), in a similar manner to dynamin I (Clayton et al. 2009). Perturbation of these specific phosphorylation sites also modulated reserve pool SV mobilisation at intense, but not mild stimulation frequencies (Chi et al. 2003), suggesting another key role for activity-dependent calcineurin-mediated dephosphorylation in the SV life cycle.

Thus key players in ADBE are also essential for the mobilisation of reserve pool SVs. In addition to sharing essential molecules, these processes may also be physically linked, since a number of requisite proteins for ADBE are enriched in the reserve SV pool cluster (Shupliakov 2009). Thus mobilisation of the reserve SV pool and ADBE are closely coupled physiological processes with strong physical and molecular links.

How many types of bulk fluid phase retrieval are there?

This review has referred to ADBE as one mechanism, however it is possible that there are multiple overlapping forms of bulk fluid phase uptake utilised by different neurones depending on their particular requirements. These fluid phase endocytosis modes may share a number of molecular similarities and can be arbitrarily separated into three separate modes.

Macroendocytosis

Macroendocytosis is a membrane retrieval mode which is responsible for the internalisation of Trk receptors in neurones (Valdez et al. 2005). It is so called because the process generates pinocytic vesicles with different diameters via membrane ruffling (like macropinocytosis) but it also concentrates cargo at internalisation sites (like CME). A fluid phase uptake route almost identical to this has been observed in the growth cones of immature primary hippocampal neuronal cultures (Bonanomi et al. 2008). This uptake mode (detected by labelling with the red styryl dye FM4-64) was dependent on actin polymerisation, PI3K activity and Rac1. It also required cholesterol and the EHD protein pincher, but not clathrin (Bonanomi et al. 2008). Importantly this endocytosis mode operated in the absence of stimulation and was only present for the first week of culture, thereafter it

was replaced by activity-dependent SV endocytosis modes. Thus macroendocytosis could be an evolutionary precursor of ADBE, sharing many common molecules but not triggered by stimulation.

ADBE - fast and slow modes?

In mature central nerve terminals (both typical and atypical) ADBE displays a characteristic fast triggering and termination during elevated neuronal activity (Cousin 2009). However a significant subpopulation of studies have reported a kinetically different form of bulk endocytosis in non-mammalian preparations. It is activity-dependent and triggered rapidly, however in contrast to mammalian nerve terminals this mode persists for minutes after termination of stimulation, with large cisternae remaining attached to the plasma membrane (Koenig and Ikeda 1989;Koenig and Ikeda 1996;Gad et al. 1998;Richards et al. 2000;Teng and Wilkinson 2000). This putative "slow" mode of ADBE could reflect a form of retrieval that has been discarded through evolution or alternatively be a consequence of the method of stimulation of these tissues (which has tended to be prolonged depolarization). To date these modes share a similar molecular mechanism (Koenig and Ikeda 1989;Richards et al. 2004;Andersson et al. 2008) suggesting that the kinetic differences observed may purely reflect triggering by non-physiological stimuli.

Perspectives

ADBE has previously been dismissed as an emergency response to excessive non-physiological stimuli in nerve terminals. However recent research has shown that this SV endocytosis mode is physiological and that it plays a key role in the nerve terminal's response to increased neuronal activity. There are many questions still be to addressed however. For example do different intensities of stimulation trigger ADBE in tonic and phasic synapses? Synapses adapted to tonic activity can support neurotransmitter release over a prolonged period of time, whereas release from phasic synapses quickly becomes depressed (Atwood and Karunanithi 2002). ADBE has been observed in phasic but not tonic synapses during identical stimuli (Evergren et al. 2006), suggesting that different nerve terminals utilise divergent SV retrieval modes that are adapted to their specific functions. Possible molecular mechanisms for these differences include a higher expression of SV endocytosis proteins in tonic synapses (Evergren et al. 2006), a differential localisation of calcineurin with the active zone, or differences in the ability of these nerve terminals to buffer calcium influx (Millar et al. 2005).

It is also anticipated that ADBE will play a major role in other physiological events that rely on elevated neuronal activity. For example ADBE has been proposed to be a compensatory retrieval mode to recapture vesicle membrane that had previously by compound SV exocytosis (He et al. 2009). In addition memory generation produced by long-term potentiation should be regulated by the extent of ADBE occurring in the nerve terminal, since this event is triggered by intense tetanic stimulation. Therefore manipulation of ADBE in nerve terminals may have acute effects on both long-term potentiation and memory. Similarly, ADBE should also play a key role in the nerve terminal response to neurological disorders such as epilepsy, where short bursts of activity release excess neurotransmitter and cause localised brain injury due to excitotoxicity. It is possible that compounds which increase the activity of ADBE could be anti-epileptic, since SVs generated by this retrieval mode will take longer to rejoin the recycling SV pool (Richards et al. 2000;Evans and Cousin 2007;Clayton et al. 2009) and thus would be unavailable for fusion. Reciprocally, pharmacological compounds that specifically inhibit CME could be anti-epileptic by directing more SV retrieval though the ADBE mode.

Thus the development of specific molecular tools to probe the key physiological and patho-physiological roles of ADBE would be highly beneficial. Development of such tools will require the discovery of specific molecular targets that are unique to ADBE. This process has begun, with the identification of proteins such as syndapin and the DAG lipase Rolling Blackout (Andersson et al. 2008;Clayton et al. 2009;Vijayakrishnan et al. 2009). Future research will provide a molecular understanding of how both of these proteins and others still be identified perform their function in ADBE in order to initiate the design of specific pharmacological inhibitors of this key SV retrieval mode.

Acknowledgments

Abbreviations

SV	synaptic vesicle
CME	clathrin-mediated endocytosis
ADBE	activity-dependent bulk endocytosis
HRP	horseradish peroxidase
BAR	bin-amphiphysin-RVS
N-WASP	neuronal Wiskott-Aldrich syndrome protein
PI3K	phosphatidyl inositol 3-kinase
PI(4,5)P$_2$	phosphatidyl inositol (4,5) bisphosphate
PIPKIγ	phosphatidyl inositol (4) phosphate 5 kinase type Iγ
EHD	Eps15 homology domain

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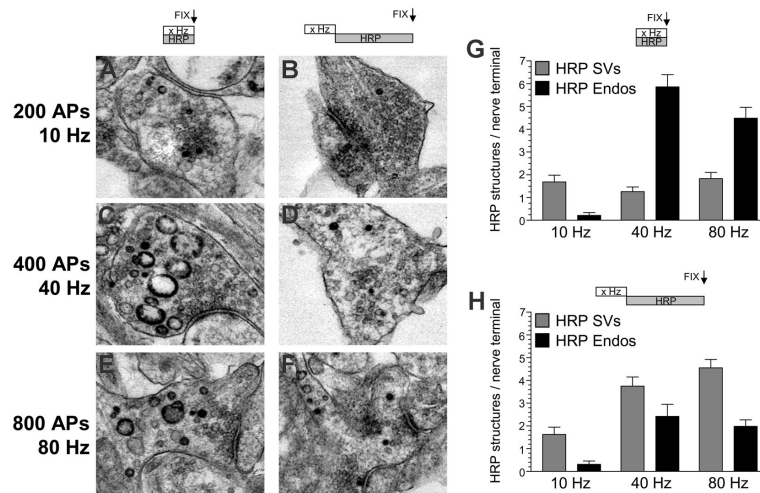
Figure 1. Activity-dependent selection of different SV retrieval modes in central nerve terminals
HRP was applied to primary neuronal cultures during trains of either 200 (A, 10 Hz), 400 (C, 40 Hz) or 800 (E, 80 Hz) action potentials and then immediately fixed. Alternatively HRP was applied to cultures for 5 min immediately after trains of either 200 (B, 10 Hz), 400 (D, 40 Hz) or 800 (F, 80 Hz) action potentials and then fixed. Representative electron micrographs are displayed (A-F). The number of HRP-labelled SVs (grey bars) or HRP-labelled endosomes (black bars) are diplayed that were generated either during (G) or after (H) stimulation is displayed. Note that with increasing stimulation intensity the number of HRP-labelled SVs does not increase, whereas at a certain threshold the number of HRP-labelled endosomes increases greatly. Also after stimulation note that the dominant SV retrieval mode is CME and not ADBE. From Clayton et al (2008) reproduced with permission, by the Society for Neuroscience.
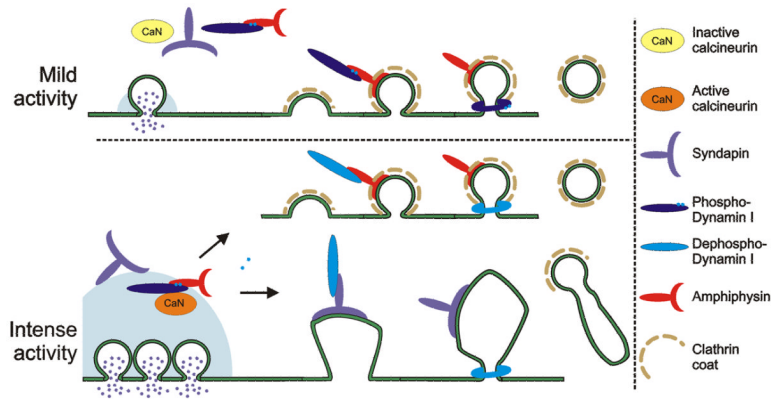
Figure 2. Selection of different SV retrieval modes by activity-dependent dynamin I dephosphorylation

During mild neuronal activity increases in intracellular free calcium are restricted to the active zone. This results in only a few SVs fusing with the plasma membrane and no activation of cytosolic calcineurin. CME can still proceed however, since phospho-dynamin I binds to amphiphysin irrespective of its phosphorylation status. Intense neuronal activity leads to increases in intracellular free calcium outside the active zone, due to the build up of residual calcium in the nerve terminal. This triggers both increased SV fusion and activation of cytosolic calcineurin. Active calcineurin then dephosphorylates dynamin I allowing an interaction with syndapin in addition to its phospho-independent interaction with amphiphysin. Thus two SV retrieval modes are now triggered, CME (which is dependent on the dynamin - amphiphysin interaction) and ADBE (which is dependent on the dynamin - syndapin interaction). Therefore both SV retrieval modes are dynamin-dependent, however only ADBE is dependent on the dephosphorylation of dynamin I.