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## Activity-Dependent Bulk Synaptic Vesicle Endocytosis - a Fast, High Capacity Membrane Retrieval Mechanism

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### Abstract

Central nerve terminals are placed under considerable stress during intense stimulation due to large numbers of synaptic vesicles (SVs) fusing with the plasma membrane. Classical clathrin-dependent SV endocytosis cannot correct for the large increase in nerve terminal surface area in the short term, due to its slow kinetics and low capacity. During such intense stimulation an additional SV retrieval pathway is recruited called bulk endocytosis. Recent studies have shown that bulk endocytosis fulfils all of the physiological requirements to remedy the acute changes in nerve terminal surface area to allow the nerve terminal to continue to function. This review will summarise the recent developments in the field that characterise the physiology of bulk endocytosis which show that it is a fast, activity-dependent and high capacity mechanism that is essential for the function of central nerve terminals.

### Keywords

Endocytosis; bulk endocytosis; clathrin; synaptic vesicle; nerve terminal; activity; calcium

### Introduction

Neurotransmission relies upon the fusion of neurotransmitter-containing synaptic vesicles (SVs) with the plasma membrane of the nerve terminal. However it is essential that this supply of SVs is maintained, otherwise neurotransmission will cease. A typical central nerve terminal only has a pool of 100 - 200 SVs and any reduction in the size of this pool will impact on the fidelity of neurotransmission, with serious consequences for brain function. To maintain supply the neuron can either generate new SVs or reuse the SVs already present in the nerve terminal. The first option is not feasible, since the nerve terminal is relatively distant from the neuronal cell body in cellular terms, meaning *de novo* generation of SVs via the endoplasmic reticulum and golgi apparatus cannot keep up with the continual requirement for neurotransmission. Instead the second option, a local recycling of SVs at nerve terminal is employed. The first step of the recycling process is the endocytosis of SV membrane and proteins from the nerve terminal plasma membrane, followed by refilling with neurotransmitter and localised transport back to sites of exocytosis.

There are multiple ways a SV can be retrieved from the nerve terminal plasma membrane. The best characterised route in both kinetic and molecular terms is clathrin-dependent endocytosis (Figure 1). In this pathway a SV is generated *de novo* from the plasma membrane using a number of molecules involving clathrin, adapter and accessory proteins

[1;2]. Clathrin-dependent endocytosis is the dominant mechanism of SV retrieval during mild neuronal activity in central nerve terminals [3]. It is a relatively slow process however, with recent studies showing that it occurs in central nerve terminals with a single time constant of approximately 14 sec [3;4].

An alternative route of SV retrieval is kiss-and-run, where the SV transiently fuses with the nerve terminal membrane before retrieving intact. This mechanism was originally proposed following studies at the frog neuromuscular junction (NMJ) in the 1970s [5;6] and occurs in a number of large secretory cells [7-9]. Kiss-and-run is a retrieval route that would be beneficial for the nerve terminal both kinetically and energetically, however its existence has been difficult to prove experimentally in neurons. This has been mainly due to the fact that all methods that attempt to monitor kiss-and-run in neurons are indirect [10].

A third mechanism of SV membrane retrieval also occurs in central nerve terminals called bulk endocytosis. This endocytosis route differs from the previous two retrieval pathways in that it does not retrieve single SVs, but rather invaginates large areas of plasma membrane to form endosomes (Figure 1). SVs are then generated from these endosomes to form a new population of SVs [11;12]. In contrast to the other two SV retrieval routes, bulk endocytosis is only triggered during elevated neuronal activity and very little is known about its molecular mechanism. What is becoming increasingly apparent however is that bulk endocytosis is a major SV retrieval route in the synapse during physiological stimulation and is essential in ensuring that the nerve terminal remains functional during periods of elevated neuronal activity. This review will discuss the role of bulk endocytosis in synaptic physiology by concentrating on the stimuli which trigger it and its time course in central nerve terminals.

## Bulk endocytosis is triggered by elevated neuronal activity

Bulk endocytosis was first described at the amphibian NMJ in a series of key morphological studies [13]. In these studies the NMJ was subjected to a massive stimulus that promoted the fusion of approximately 5000 SVs per nerve terminal. Since the applied stimulus was non-physiological, the authors proposed that bulk endocytosis may not occur during normal neurotransmission. However further studies in this preparation showed that bulk endocytosis could be stimulated by tetanic action potential stimulation [11;14]. Furthermore relatively small increases in nerve terminal activity (5 Hz for 30 s) evoked the process in the NMJ of reptiles [15]. Thus in neuromuscular preparations bulk endocytosis is triggered by stimuli within the physiological range.

What type of stimulus evokes bulk SV endocytosis in central nerve terminals? Bulk endocytosis has been widely observed in a number of nerve terminals that have different levels of background activity, SV populations and functions. These include isolated nerve terminals (synaptosomes) [16], primary neuronal cultures derived from either cerebellum, hippocampus or cortex [12;17-19], retinal bipolar neurones [20] and the Calyx of Held [21]. In all of these studies the stimulus was prolonged exposure to elevated KCl, which does not mimic physiological stimulation conditions. However other studies have shown that bulk endocytosis can also be triggered by brief trains of action potentials equivalent to those encountered *in situ* by neurones. For example, in retinal bipolar neurones the fluid phase marker ferritin was accumulated into bulk endosomes during brief depolarizations caused by calcium-dependent action potentials [22]. In the Calyx of Held, individual bulk endocytosis events (monitored by membrane capacitance) were triggered by 1 second of 10 Hz stimulation [23]. Finally in central nerve terminals in primary neuronal culture, brief trains of action potentials were sufficient to stimulate bulk endocytosis, monitored by either the uptake of large fluorescent dextrans (which are too large to be accumulated inside SVs) or

by morphological analysis of the uptake of the fluid phase marker horse radish peroxidase (HRP) [24]. Thus in both neuromuscular preparations and in central nerve terminals bulk endocytosis is triggered by stimuli within the physiological range. This places bulk endocytosis as a key retrieval route during elevated neuronal activity.

## Bulk endocytosis is triggered immediately by neuronal activity

Increases in the level of neuronal activity within the physiological range trigger bulk endocytosis in central nerve terminals. However the speed at which bulk endocytosis is activated has not been widely studied. The balance of experimental evidence suggests that bulk endocytosis is triggered co-incident with increased neuronal activity. For example, in the original studies at the frog NMJ, bulk endocytosis was one of the first events to be observed - within one second of stimulation [13]. Also in reptile NMJs, large fluorescent dextrans were accumulated into nerve terminals within 1-2 seconds of stimulation [25]. In central neurones these rapid kinetics are also observed. For example in retinal bipolar neurones, the majority of the fluid phase marker ferritin was accumulated into endosomes within seconds of the initiation of calcium-dependent action potentials [22]. In primary cultures of cerebellar granule neurones and in isolated nerve terminals, bulk endosomes were abundant within 5 to 15 seconds of stimulation with elevated KCl [16;17] or immediately after brief (10 sec) trains of action potentials [24]. Finally in electrophysiological studies in the Calyx of Held, individual bulk endocytosis events were observed less than 1 second after initiation of stimulation [23]. Thus bulk endocytosis is triggered rapidly after the onset of elevated neuronal activity, indicating that it will be an active and key participant in SV retrieval during these stimulation conditions.

## Mechanism of bulk endocytosis triggering

Studies in central nerve terminals have shown that the number of SVs retrieved by clathrin-dependent endocytosis do not scale in parallel with increased neuronal activity, suggesting that this retrieval route has a limited capacity [24;26]. This provides a compelling reason for the activation of bulk endocytosis by elevated neuronal activity - to provide additional capacity for SV membrane retrieval. Saturation of the clathrin-dependent endocytosis machinery may trigger bulk endocytosis, however there is little direct evidence to indicate that this is the case. What is more likely is that bulk endocytosis is triggered by elevated neuronal activity itself. The most obvious reporter of neuronal activity is calcium influx via voltage-dependent calcium channels, since this will accurately communicate the extent of stimulation within individual nerve terminals. Following this logic a calcium-dependent sensor must be present in nerve terminals that triggers bulk endocytosis. In theory this sensor should not be activated by levels of calcium influx evoked by mild neuronal activity, but should be activated by calcium increases evoked by elevated neuronal activity. One such activity-dependent sensor is the calcium-dependent protein phosphatase calcineurin.

Calcineurin is an excellent candidate for the activity-dependent bulk endocytosis trigger for a number of reasons. First it has an affinity for calcium in the low micromolar range [27], therefore it would not detect calcium increases due to mild activity. Second it is located in the cytosol, so it would only encounter calcium increases produced by trains of action potentials that can transiently override the calcium buffering systems of the nerve terminal. Finally and most importantly, it is activated by the same stimuli that trigger bulk endocytosis. Our group have shown that the dephosphorylation of the calcineurin substrate dynamin I only occurs at the levels of neuronal activity that trigger bulk endocytosis in primary neuronal culture (unpublished observations). Thus calcineurin has an activation threshold in nerve terminals, which is tuned to the same levels of activity that trigger bulk endocytosis. The role for calcineurin as the activity sensor for bulk endocytosis is supported

by studies showing that it is only required for SV retrieval that is evoked by elevated, but not mild, neuronal activity in a number of neuronal systems [12;28-30]. Thus calcineurin is perfectly placed in the nerve terminal to be the activity-dependent trigger for bulk endocytosis. In agreement its inhibition arrests bulk endocytosis in primary neuronal culture [12]. Further studies will be required to determine whether the dephosphorylation of some or all of its nerve terminal substrates (the dephosphins [31]) are essential for bulk endocytosis.

## **Bulk endocytosis is the dominant SV retrieval route during elevated neuronal activity**

As discussed above, bulk endocytosis provides a high capacity mechanism to retrieve large amounts of SV membrane during elevated neuronal activity. However some studies have suggested that bulk endocytosis also persists for minutes after neuronal activity has ceased. One such study was performed in the frog NMJ. In this study, lipid binding dyes of different hydrophobicity (FM1-43 and FM2-10) were used to label endocytosis routes that were active either during or after tetanic stimulation [11]. When dyes were applied during tetanus, more FM1-43 was accumulated into bulk endosome cisternae than FM2-10. The authors suggested this difference arose from the more hydrophilic FM2-10 being washed out of cisternae that were still attached the plasma membrane when stimulation terminated. To test this theory, FM2-10 was added after stimulation and an additional component of dye uptake was observed. This labelling took approximately 15 minutes to complete, suggesting that bulk endocytosis did persist for minutes after strong stimulation. However this post-stimulation retrieval route may be unrelated to bulk endocytosis for a number of reasons. For example, FM1-43 did not label this post-stimulation endocytosis route under identical conditions, and no morphological analysis was performed on the structures labelled by FM2-10 after stimulation. In agreement it was recently shown that the ability of these dyes to selectively label bulk endocytosis was due to their affinity for membrane and not their washout properties [30].

Other studies have shown that large cisternae were still attached to the plasma membrane for tens of minutes after removal of an endocytosis block [32-34], suggesting bulk endocytosis persists after termination of stimulation. However since these preparations were subjected to extreme treatment (i.e. complete SV depletion), it is difficult to extrapolate these results in relation to the physiological time course of bulk endocytosis.

Recent studies in central nerve terminals using physiological stimulation have shown that bulk endocytosis does not persist when elevated neuronal activity ceases. First, capacitance measurements of bulk endocytosis in the Calyx of Held show that scission of bulk endosomes occurs within 10 seconds of their formation [23], arguing against a prolonged attachment to the plasma membrane. Furthermore, large fluorescent dextrans were not internalised when they were applied immediately after delivery of a brief train of action potentials to small central nerve terminals in culture [24], suggesting bulk endosomes had already detached from the plasma membrane. The most informative information has come from morphological studies examining the uptake of HRP that was applied either during or after a brief train of action potentials [24]. In these studies the great majority of bulk endocytosis events occurred during the 10 second stimulus rather than afterwards. This was in stark contrast to HRP labelling of SVs, where the majority of SVs were internalised after the 10 second stimulus had terminated [24]. These results are in close agreement with the initial studies in the frog NMJ, which showed that bulk endocytosis arrested very quickly after termination of stimulation, whereas clathrin-dependent SV endocytosis continued for minutes afterwards [13].

Thus bulk endocytosis is the dominant SV retrieval route during brief physiological trains of action potentials. In agreement with its calcium-dependent triggering, bulk endocytosis arrests very quickly after action potential stimulation terminates. The opposite is true for clathrin-dependent SV endocytosis, which persists for minutes after stimulation and is thus the dominant SV retrieval route after elevated neuronal activity ceases.

## Perspectives

Recent experimental evidence in central nerve terminals provides a clear description of bulk endocytosis as a high capacity, activity-dependent retrieval route that is immediately triggered by elevated neuronal activity and which immediately shuts down when activity ceases. As discussed some studies contradict this model of a fast, rapidly inactivating process. There are a number of explanations for this, the simplest being that multiple pathways are being reported under the global banner of bulk endocytosis. In support, two different forms of bulk endosome have been reported in the lizard NMJ, one formed immediately after brief intense trains, and another that had deep invaginations contiguous with the extracellular space [15]. The morphology of bulk endosomes also seem to differ between different systems, with flattened cisternae observed in amphibian NMJs [11], while in central nerve terminals round endosomes are observed [12;18;19;24]. Another possible reason for discrepancies may be the extreme manipulations of the neuronal systems used by researchers in some cases. For example a form of bulk endocytosis with different kinetic properties has been observed when nerve terminals recover from a complete depletion of SVs caused by either genetic [32;35;36] or pharmacological [34] treatments. The large invaginations observed during these conditions may be due to the recruitment of a stress-dependent macropinocytic pathway which is triggered as an emergency measure. The elucidation of the molecules that participate specifically in bulk endocytosis will help greatly to differentiate between these pathways and allow a more systematic examination of their role in different neuronal systems.

The description of bulk endocytosis as an activity-dependent, high capacity membrane retrieval mechanism has large implications for the study of nerve terminal physiology. First it provides an explanation as to how small central nerve terminals retain their structure and function during times of intense neuronal activity, by supplying an additional retrieval route to recover excess SV proteins and membrane deposited by increased exocytosis. These SVs are not likely to be immediately available for fusion however, rather they selectively replenish the reserve SV pool [12]. It also highlights that by definition bulk endocytosis must play a key role in a number of physiological processes that are dependent on elevated neuronal activity. These include processes such as long-term potentiation and adaptation, which result in both long and short term changes in synapse strength. Bulk endocytosis will also be a major component of the nerve terminal response to certain pathophysiological events, such as to epileptic burst firing. In summary after years of neglect by researchers, bulk endocytosis is starting to emerge as a critical retrieval route in physiological response to elevated neuronal activity by central nerve terminals. Now that its physiology has been characterised the next five years should see a concerted effort to understand its molecular mechanism.

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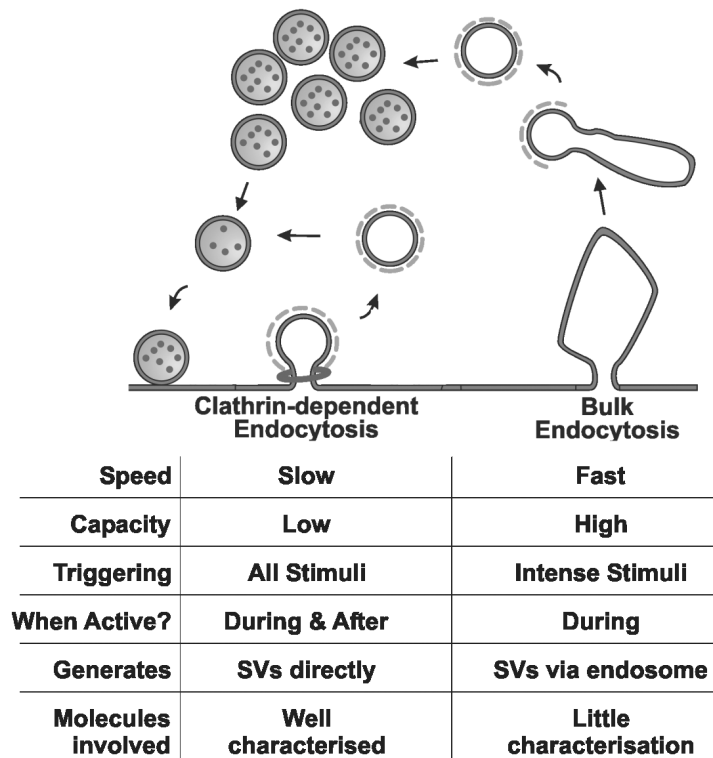
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**Figure 1.**  
Comparison of bulk endocytosis with clathrin-dependent endocytosis.