Fine-tuning activity-dependent bulk endocytosis via kinases and phosphatases

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Title: **Fine-tuning activity-dependent bulk endocytosis via kinases and phosphatases**

Running title: **Phospho-regulation of synaptic vesicle endocytosis**

eTOC: **Bidirectional phosphorylation-dependent control of two discrete synaptic vesicle endocytosis modes via calcineurin and minibrain kinase**

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The regulation of activity-dependent bulk endocytosis, the dominant mode of membrane retrieval in response to intense neuronal activity, is poorly understood. In this JCB issue, Geng *et al*. (xxxx) propose a novel molecular mechanism for the coordination of activity-dependent bulk endocytosis that builds on Minibrain kinase and its presynaptic substrate synaptojanin-1.

Subjects: brain activity regulation, neurotransmission, membrane trafficking, synaptic vesicle recycling

Brain function necessitates sustained synaptic transmission regardless of activity demands. The preservation of synaptic transmission depends on the efficient (re)formation of synaptic vesicles (SVs) by endocytosis after their insertion into the synaptic plasma membrane during neuronal stimulation (1). During mild and sparse stimulation, the dominant endocytosis modes are ultrafast endocytosis and clathrin-mediated endocytosis (CME) (1). Both modes appear to have a fixed rate and limited capacity, and therefore cannot adapt to high frequency stimulations that accumulate inserted SV membranes at the presynaptic terminal. Under these conditions, a different endocytosis mode is predominantly utilized, termed activity-dependent bulk endocytosis (ADBE). ADBE retrieves large areas of the presynaptic plasma membrane to form bulk endosomes, from which new SVs are then generated (1). This form of endocytosis is particularly common in synapses that operate with high rates of neurotransmission, e.g., ribbon synapses of sensory neurons. ADBE contributes to presynaptic plasticity, having recently been demonstrated to control neurotransmitter release probability (2). Importantly, defects in ADBE and SV endocytosis in general have profound consequences on neuronal function and survival, with dysfunction linked to a series of neurodevelopmental disorders (3).

Considering the importance of ADBE to brain physiology and pathology, it is essential to understand the molecular machinery that controls this process and synchronizes it with other synaptic events. Amazingly, despite the fact that ADBE was described in the early 1970s, its regulation remains mysterious. Several protein kinases and phosphatases that contribute to regulation of clathrin-mediated endocytosis and other endocytosis modes (1), may also contribute to ADBE. For example, the calcium/calmodulin-dependent phosphatase calcineurin activates ADBE, working with glycogen synthase kinase-3 (GSK-3) to provide bidirectional control via the phosphorylation of specific substrates (4). However many presynaptic proteins are calcineurin substrates, suggesting other protein kinases may perform complementary roles.

In a recent paper, Chang and colleagues (xxxx) present data in support of calcineurin and Minibrain/DYK1A (Mnb) as co-regulators of ADBE in fruit flies via bidirectional control of the phosphorylation status of synaptojanin-1 phosphatase. The authors argue that the synaptojanin-1 phosphorylation status coordinates the activity-dependent balance between CME vs. ADBE. Namely, during mild stimulation CME is promoted by Mnb, while ADBE is inhibited. During intense stimulation, dephosphorylation of synaptojanin-1 by calcineurin is required to activate ADBE. An interesting novel aspect arises from examination of domain-specific synaptojanin-1 mutants: its 4'-phosphatase SAC1 activity supports ADBE, while its 5'-phoshatase domain suppresses it. The N-BAR domain protein endophilin-A has been implicated in ADBE (5), however a synaptojanin-1 mutant lacking the endophilin-A binding PRD domain had no effect. Further studies may therefore be required to dissect synaptojanin-1-dependent and -independent roles of endophilin in ADBE.

Collectively, the data by Chang and colleagues consolidate the key role played by calcineurin in ADBE and identify Mnb as a new ADBE protein kinase. Intriguingly, the number of synapses performing ADBE is increased in Mnb hypomorphs, suggesting there is additional endocytic capacity that can be recruited on demand. There also appears to be bidirectional control of ADBE via Mnb, since Mnb overexpression represses this pathway. Notably, the enzyme activities of synaptojanin-1 are regulated by Mnb- and calcineurin-dependent turnover of phosphorylation of S1029 (6,7). In mammals, cyclin-dependent kinase 5 (Cdk5) is suggested to control synaptojanin-1 activity (8), therefore it important to confirm whether synaptojanin-1 is also phosphorylated by the Mnb orthologue, dual specificity tyrosine-phosphorylation-regulated kinase (DYRK1A), in mammals. A key test of the causality of activity-dependent phosphorylation events is whether they occur to the same stimulation intensities as the biological event. In this study, activity-dependent dephosphorylation of S1029 on synaptojanin-1 was not demonstrated, instead an absence of activity-dependent Mnb phosphorylation was observed. In mitigation, the authors convincingly demonstrated that synaptojanin-1 phosphorylation increased during prolonged stimulus in the absence of calcineurin function.

This work also confirmed a key role for the phospholipid $PI(4,5)P_2$ in ADBE (1). Interestingly, it further revealed a hitherto undiscovered role for the SAC, but not the 5'-phosphatase, domain of synaptojanin-1 in ADBE. This latter activity is essential for other forms of endocytosis, such as clathrin-mediated and ultrafast endocytosis, with SAC activity required for clathrin-dependent vesicle generation from endosomes (9,10). In addition to potential roles for synaptojanin-1 SAC activity discussed by Chang and colleagues, a more provocative (and simplistic) explanation is that the end product, phosphatidylinositol (PI) itself, is important for ADBE. In support, the neurons without DAG kinase (which generates the PI precursor phosphatidic acid) display SV endocytosis defects that are exacerbated during high activity (11).

A lack of accurate assays that monitor ADBE in both time and space has limited research in small nerve terminals for decades. In this work, ADBE is evoked and monitored using multiple approaches. This is important, since there is no simple method to monitor ADBE, therefore it requires cross corroboration wherever possible. This study was greatly assisted via the use of genetically-tractable model organisms, allowing precise intervention to abate the function of key proteins and enzymes *in vivo*. Yet, the trade-off is the relative imprecision of stimulation to evoke

SV turnover, with prolonged periods of stimulation (and parallel inhibition of CME) required to evoke and isolate ADBE.

Since Geng *et al*. shed light on new aspects of ADBE regulation, further questions can now be envisioned. In particular, how localised production and degradation of membrane phospholipids coordinate the temporal and spatial triggering of specific endocytosis modes. The essential role for calcineurin in most forms of endocytosis suggests where and when dephosphorylation events occur at the presynapse may be critical in the recruitment of discrete SV reformation pathways. Furthermore, Mnb/DYRK1A is linked to brain pathologies, including Down syndrome and autism-spectrum disorders, which is yet to be explored. These and other questions will no doubt drive further studies of remarkable plasticity when it comes to formation of new SVs and synaptic transmission, and how they organize and govern our brain activity.

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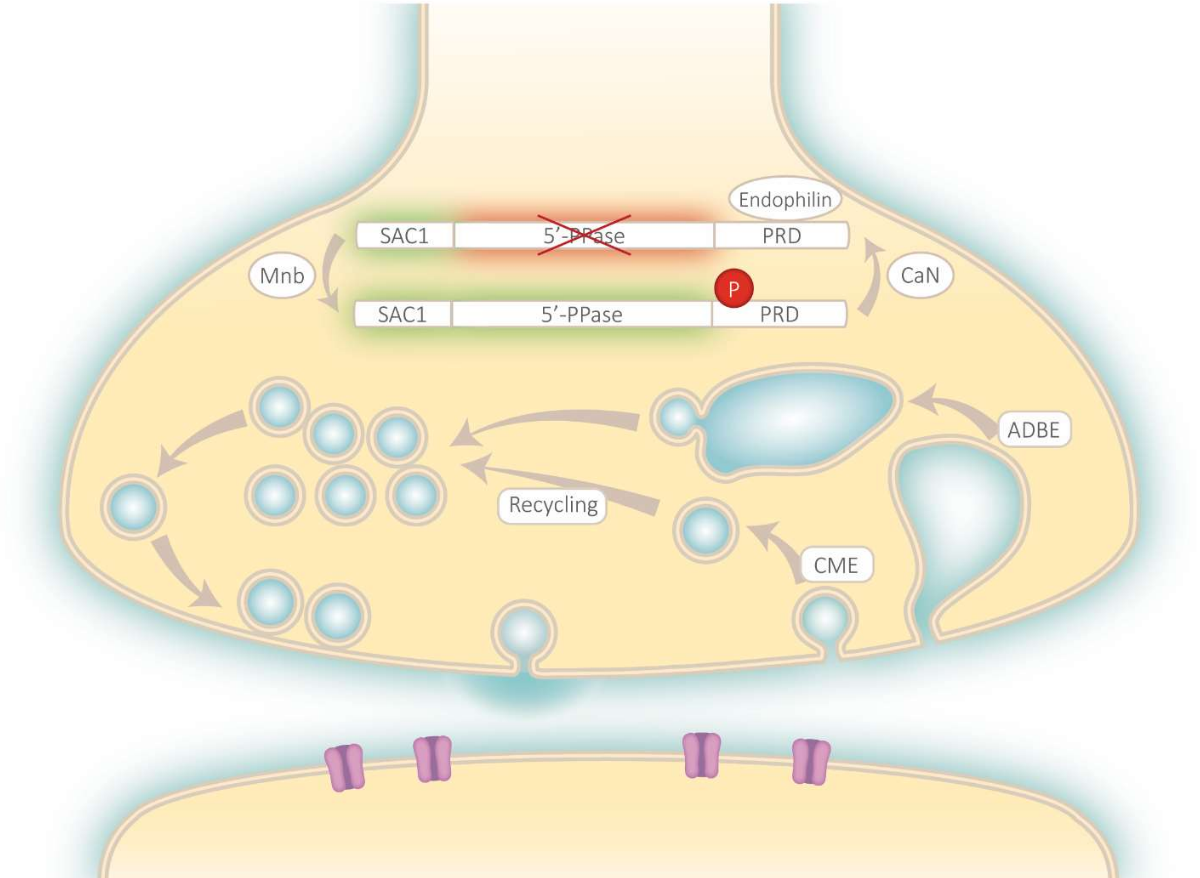

Figure Legend Synaptojanin (Synj) is phosphorylated by minibrain (Mnb) kinase on Ser1029 on its proline-rich domain (PRD). This promotes the 5'-phosphatase (5'-PPase) activity of Synj, and inhibits association with the endocytosis protein endophilin. These events promote clathrin-mediated endocytosis (CME). During intense neuronal activity, calcineurin (CaN) is activated and dephosphorylates Synj. This reduces 5'-PPase activity, and promotes association with endophilin. This dephosphorylation also promotes activity-dependent bulk endocytosis (ADBE) via inhibition of Synj 5’-PPase activity. This phospho-regulation of the endophilin interaction does not impact ADBE. The SAC activity of Synj is essential for ADBE, and is unaffected by phosphorylation.